Rooibos and Melatonin: Putative modulation of nicotine-induced effects on vascular function

by
Michelle Smit-van Schalkwyk

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Pectora roborant cultus recti

Supervisor: Prof Hans Strijdom
Co-supervisor: Dr Shantal Windvogel

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Introduction and aims: Cigarette smoking is an important risk factor for cardiovascular disease, and nicotine, the addictive substance in tobacco, is implicated in the pathogenesis of atherosclerosis via oxidative stress. Oxidative stress can lead to loss of vascular homeostasis, via reduced nitric oxide (NO) bioavailability, which in turns leads to endothelial dysfunction. Endothelial dysfunction is an early, reversible precursor of atherosclerosis and cardiovascular disease. This dissertation aimed to investigate whether treatment with the indigenous South African plant rooibos (fermented and unfermented) and the hormone melatonin can ameliorate the harmful effects of nicotine on the vascular and endothelial systems.

Methodology: This study made use of an *in vivo* model (male Wistar rats) and *ex vivo* model (rat aortic ring segments), complemented by an *in vitro* model of cultured rat aortic endothelial cells (AECs). Biometric data, fluid intakes, blood pressure measurements, vascular function, antioxidant/oxidative stress status, inflammatory markers and serum lipids were determined as measures of the effects of nicotine (5 mg/kg bw/day; subcutaneously administered) in the rat model. The effects of nicotine co-treated with rooibos (2% administered as drinking fluid) and nicotine co-treated with melatonin (4 mg/kg bw/day; administered as drinking fluid) were assessed according to the same endpoints to determine if the nicotine-induced effects observed could be ameliorated. The modulating effects of fermented rooibos (RF) were further investigated in an *in vitro* model of AECs. Nicotine injury (100 µM over a 24 hour treatment period) was assessed by means of NO production and cell viability assays. The effects of nicotine, pre-treated with RF (0.015 mg/ml) were assessed according to the same endpoints.

Results: *Nicotine-induced vascular injury:* Nicotine treatment (six weeks) in rats exerted significant effects on biometric parameters, reduced fluid consumption, increased blood pressure, and elicited pro-contractile responses in aortic rings. Furthermore, nicotine administration led to a decrease in superoxide dismutase (SOD) activity (liver) and catalase (CAT) activity (heart and liver), and increased lipid peroxidation. The harmful vascular effects of nicotine were further underscored by reduced intracellular NO levels and reduced cell viability in AECs. *Nicotine and rooibos co-treatment:* RF exerted anti-contractile and pro-relaxation responses in aortic rings and increased SOD and CAT activity (liver) in nicotine-treated animals. RF pre-treatment led to increased intracellular NO levels in nicotine-treated AECs. Unfermented rooibos (RUF) exerted anti-contractile responses in aortic rings and increased CAT activity (heart) in nicotine-treated animals. *Nicotine and melatonin co-treatments:* Melatonin reduced blood pressure, exerted anti-contractile and pro-relaxation

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responses in aortic rings, increased SOD activity (heart) and CAT activity (heart and liver), as well as decreased lipid peroxidation in nicotine-treated animals.

Conclusion: Nicotine administration resulted in significant vascular and endothelial injury, associated with increased oxidative stress and reduced antioxidant activity. Overall, our data showed that rooibos, specifically RF, and melatonin exerted beneficial effects on the vascular and endothelial system of nicotine exposed rats. Mechanisms identified in this dissertation included their antioxidant properties, although other mechanisms cannot be ruled out. Restoration of vascular homeostasis, underscored by eNOS activation and subsequent release of NO, could underlie the modulating actions of both rooibos and melatonin.

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OPSOMMING

Inleiding en doelwitte: Sigaretrook is ‘n belangrike risikofaktor vir kardiovaskulêre siekte, en nikotien, die verslawende substans in tabak, word met die patogenese van aterosklerose via oksidatiewe stres, geassosieer. Oksidatiewe stres kan lei tot ‘n verlies aan vaskulêre homeostase via verlaagde stikstofoksied (NO) biobeskikbaarheid, wat tot endodeeldisfunksie aanleiding kan gee. Endoteeldisfunksie is ‘n vroeë, omkeerbare voorloper van aterosklerose en kardiovaskulêre siekte. Die doel van hierdie proefskrif was om te bepaal of behandeling met die inheemse Suid-Afrikaanse plant, rooibos (gefermenteer of ongefermenteer) en die hormoon, melatonien, die skadelike effekte van nikotien op die vaskulêre en endoteel stelsels kan teenwerk.

Metodes: Daar is van ‘n *in vivo* model (manlike Wistar rotte) en *ex vivo* model (aorta ring segmente) gebruik gemaak, sowel as ‘n *in vitro* model van rot aorta endoteelselle (AECs). Biometriese data, vloeistof inname, bloeddruk, vaskulêre funksie, antioksidant/oksidatiewe stres status, inflammatoriese merkers en serum lipiede is gemeet as eindpunte van die effekte van nikotien (5 mg/kg liggaamsgewig/dag; subkutaan toegedien) in die rot model. Die gevolge van gesamentlike toediening van nikotien en rooibos (2% toegedien in drinkwater) en nikotien en melatonien (4 mg/kg liggaamsgewig/dag; toegedien in drinkwater) was vervolgens gemeet om te bepaal of die nikotien-geïnduseerde effekte teengewerk kon word. Die modulerende effekte van gefermenteerde rooibos (RF) is verder ondersoek in ‘n *in vitro* model van AECs. Nikotien besering (100 µM oor 24 uur) is deur middel van intrasellulêre NO-produksie en sellewensvatbaarheid metings bepaal. Die gevolge van nikotien, vooraf behandel met RF (0.015 mg/ml) is beoordeel volgens dieselfde eindpunte.

Resultate: *Nikotien-geïnduseerde vaskulêre besering:* Nikotientoediening (ses weke) het ‘n beduidende uitwerking op biometriese eindpunte gehad, vloeistof inname verminder, bloeddruk verhoog en ‘n pro-kontraktiele respons in aorta ringe ontlok. Nikotientoediening het ook superoksied dismutase (SOD) en katalase (CAT) aktiwiteit (hart en lewer) verlaag, en lipiedperoksidasie verhoog. Die skadelike vaskulêre effekte van nikotien is bevestig deur die waarneming van verlaagde intrasellulêre NO-vlakke en sellewensvatbaarheid in nikotien-behandelde AECs. *Gesamentlike nikotien en rooibos toediening:* RF het anti-kontraktiele en pro-dilaterende effekte in aorta ringe, asook verhoogde SOD en CAT aktiwiteit (lewer) in nikotien-behandelde diere tot gevolg gehad. Vooraf behandeling met RF het gelei tot verhoogde intrasellulêre NO-vlakke in nikotien-behandelde AECs. Ongefermenteerde rooibos (RUF) het anti-kontraktiele effekte in aorta ringe en verhoogde CAT aktiwiteit (hart) in nikotien-behandelde diere tot gevolg gehad. *Gesamentlike nikotien en melatonien toediening:* Melatonien het bloeddruk verlaag, anti-kontraktiele en pro-dilaterende effekte in aorta ringe,

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verhoogde SOD aktiwiteit (hart) en CAT aktiwiteit (hart en lewer), asook verlaagde lipied peroksidasie in nikotien-behandelde diere tot gevolg gehad.

Gevolgtrekkings: Nikotientoediening het beduidende vaskulêre en endoteel skade veroorsaak, wat met verhoogde oksidatiewe stres en verlaagde anti-oksidant aktiwiteit gepaard gegaan het. Oor die algemeen toon ons resultate dat rooibos, spesifiek RF, en melatonien voordelige effekte op die vaskulêre en endoteel sisteme van nikotien-behandelde rotte gehad het. Die proefskrif het anti-oksidant aktiwiteit as ‘n moontlike meganisme geïdentifiseer wat hierdie effekte kan verklaar, alhoewel ander meganismes nie uitgesluit kan word nie. Die herstel van vaskulêre homeostase, weens eNOS aktivering en daaropvolgende vrystelling van NO, kan verder onderliggend tot die beskermende aksies van beide rooibos en melatonien wees.

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'*Somewhere, something incredible is waiting to be known*'

Carl Sagan

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List of Tables

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List of Figures

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List of Abbreviations

5-HT	-	5-hydroxytryptamine
8-OHdg	-	8-hydroxy-2'-deoxyguanosine
AA-NAT	-	Arylalkylamine *N*-acetyltransferase
ACE 2	-	Angiotensin converting enzyme 2
ACh	-	Acetylcholine
AECs	-	Aortic endothelial cells
AMPK	-	AMP-activated protein kinase
Ang	-	Angiotensin
ARE	-	Antioxidant response element
ATP	-	Adenosine triphosphate
BCA	-	Bicinchoninic acid
BH4	-	(6R)-5,6,7,8-tetrahydrobiopterin
BHT	-	Butylated hydroxytoluene
BSA	-	Bovine serum albumin
Ca^{2+}	-	Calcium
CAT	-	Catalase
CD	-	Conjugated dienes
CNS	-	Central nervous system
CO_2	-	Carbon dioxide
COX-2	-	Cyclooxygenase-2
CRP	-	C-reactive protein
Cu	-	Copper
CVD	-	Cardiovascular disease
DAC	-	4-(dimethylamino)-cinnamaldehyde
DAD	-	Diode array detector

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DAF-2/DA	-	4,5-diaminofluorescein-2/diacetate
DEA/NO	-	Diethyl NONOate diethylammonium salt
deiH_2O	-	Deionized water
DMSO	-	Dimethyl sulfoxide
ECM	-	Extracellular matrix
ECs	-	Endothelial cells
ED	-	Endothelial dysfunction
EDHF	-	Endothelium-derived hyperpolarising factor
EDRF	-	Endothelium-derived relaxing factor
EGFR	-	Epidermal growth factor receptor
EGM-2	-	Endothelial cell growth medium
ELISA	-	Enzyme linked immunosorbent assay
eNOS	-	Endothelial nitric oxide synthase
ERK	-	Extracellular signal regulated kinase
ET-1	-	Endothelin-1
ETC	-	Electron transport chain
FAD	-	Flavin adenine dinucleotide
FBS	-	Foetal bovine serum
Fe	-	Iron
FMN	-	Flavin mononucleotide
G	-	Gauge
GABA	-	γ-aminobutyric acid
GBD	-	Global burden of disease
GPx	-	Glutathione peroxidase
GR	-	Glutathione reductase
H_2O	-	Water

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H_2O_2	-	Hydrogen peroxide
HAECs	-	Human aortic endothelial cells
HDL	-	High density lipoproteins
hEGF	-	Human epidermal growth factor
hFGF	-	Human fibroblastic growth factor
HIOMT	-	5-hydroxyindole-*O*-methyl-transferase
HO•	-	Hydroxyl radical
HUVECs	-	Human umbilical vein endothelial cells
ICAM	-	Intercellular adhesion molecule
IL	-	Interleukin
iNOS	-	Inducible nitric oxide synthase
K^+	-	Potassium
KHB	-	Krebs Henseleit buffer
LDL	-	Low-density lipoprotein
LPS	-	Lipopolysaccharide
MDA	-	Malondialdehyde
MEC	-	Mecamylamine
Mel	-	Melatonin
mmHg	-	Millimetres of Mercury
Mn	-	Manganese
MS	-	Metabolic syndrome
nACh	-	Nicotinic acetylcholine
nAChRs	-	Nicotinic acetylcholine receptors
NaCl	-	Sodium chloride
NCD	-	Non-communicable diseases
NMel	-	Nicotine and melatonin

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nNOS	-	Neuronal nitric oxide synthase
NO	-	Nitric oxide
NOS	-	Nitric oxide synthase
NRF	-	Nicotine and fermented rooibos
Nrf2	-	Nuclear factor erythroid 2-related factor
NRT	-	Nicotine replacement therapy
NRUF	-	Nicotine and unfermented rooibos
$O_2^{\bullet-}$	-	Superoxide
$OONO^-$	-	Peroxynitrite
OxLDL	-	Oxidised low-density lipoprotein
PARP	-	Poly(ADP-ribose) polymerase
PBS	-	Phosphate buffered saline
PFH	-	Perifornical hypothalamus
PGI_2	-	Prostacyclin
Phe	-	Phenylephrine
PI	-	Propidium iodide
PL	-	Phospholipid
PNS	-	Peripheral nervous system
PPAG	-	Phenylpyruvic acid-2-O-glucoside
Prx-III	-	Peroxiredoxine
R^3-IGF-1	-	Long chain human insulin-like growth factor
RAAS	-	Renin angiotensin aldosterone system
RF	-	Fermented rooibos
RNS	-	Reactive nitrogen species
ROS	-	Reactive oxygen species
RUF	-	Unfermented rooibos

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SCN	-	Suprachiasmatic nucleus
sGS	-	Soluble guanylyl cyclase
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TBARS	-	Thiobarbituric acid reactive substances
TC	-	Total cholesterol
TG	-	Triglycerides
TNF	-	Tumour necrosis factor
TXA_2	-	Thromboxane
UK	-	United Kingdom
USA	-	United States of America
UV	-	Ultraviolet
VCAM-1	-	Vascular adhesion molecule-1
VEGF	-	Vascular endothelial growth factor
VPR	-	Volume-pressure recording
VSMC	-	Vascular smooth muscle cells
VTA	-	Ventral tegmental area
WHO	-	World Health Organisation
WKY	-	Wistar-Kyoto
Zn	-	Zinc

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Units of Measurement

%	-	percentage
°C	-	degrees Celsius
µg	-	microgram
µl	-	microlitre
µM	-	micro molar
g	-	gram
mg	-	milligram
min	-	minutes
ml	-	millilitre
mm	-	millimetre

Symbols

α	-	alpha
β	-	beta
γ	-	gamma

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Table of Contents

Stellenbosch University https://scholar.sun.ac.za

Detailed Table of Contents

Stellenbosch University https://scholar.sun.ac.za

Stellenbosch University https://scholar.sun.ac.za

Stellenbosch University https://scholar.sun.ac.za

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CHAPTER 1: BACKGROUND

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1.1. Introduction

In 2011 the World Health Organisation (WHO) reported non-communicable diseases (NCD) to be the leading cause of death worldwide, with cardiovascular disease (CVD) being the leading cause of death amongst NCD (Fig 1.1) (Mendis, 2011). It is expected that cardiovascular deaths will escalate to 23.4 million by the year 2030 (World Health Organization, 2009). According to the Global Burden of Disease (GBD) 2010 report, CVD was responsible for ~30% of all-age deaths globally, and for ~9% of deaths in sub-Saharan Africa (GBD 2010). In addition, cardiovascular mortality in South Africa is on the rise, specifically in the working age group (35 – 64 years), which can lead to a negative impact on the economy of the country (Steyn, 2007). Cigarette smoking is one of the most important risk factors of CVD. Smoking is responsible for ~12% (6.2 million) of all deaths globally (Fig 1.2) (GBD 2010), and for 12% of deaths in men and 4% of deaths in women in South Africa (Groenewald *et al*., 2007). It is estimated that over five million people are current or ex tobacco users and that over 600,000 non-smokers die from the exposure to second hand smoke (World Health Organization, 2011). Nicotine, the addictive substance in tobacco, is implicated in the pathogenesis of atherosclerosis, an important precursor of CVD (Tsiara *et al*., 2003 and Kilaru *et al*., 2001).

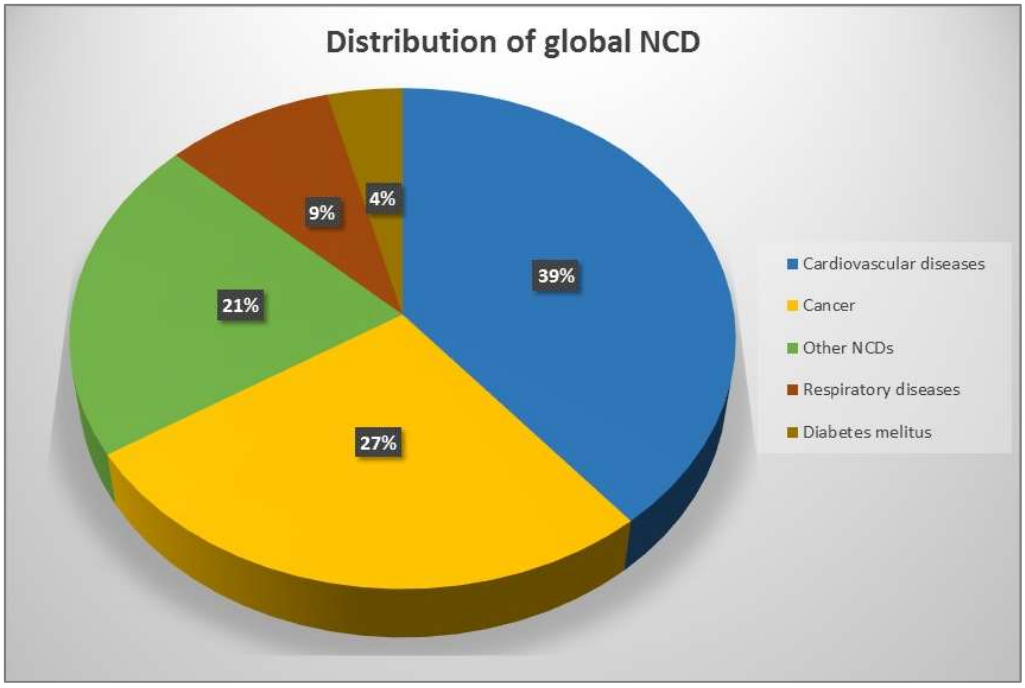

Figure 1.1. Distribution of global NCD by cause of death in persons less than 70 years old. (Mendis, 2011).

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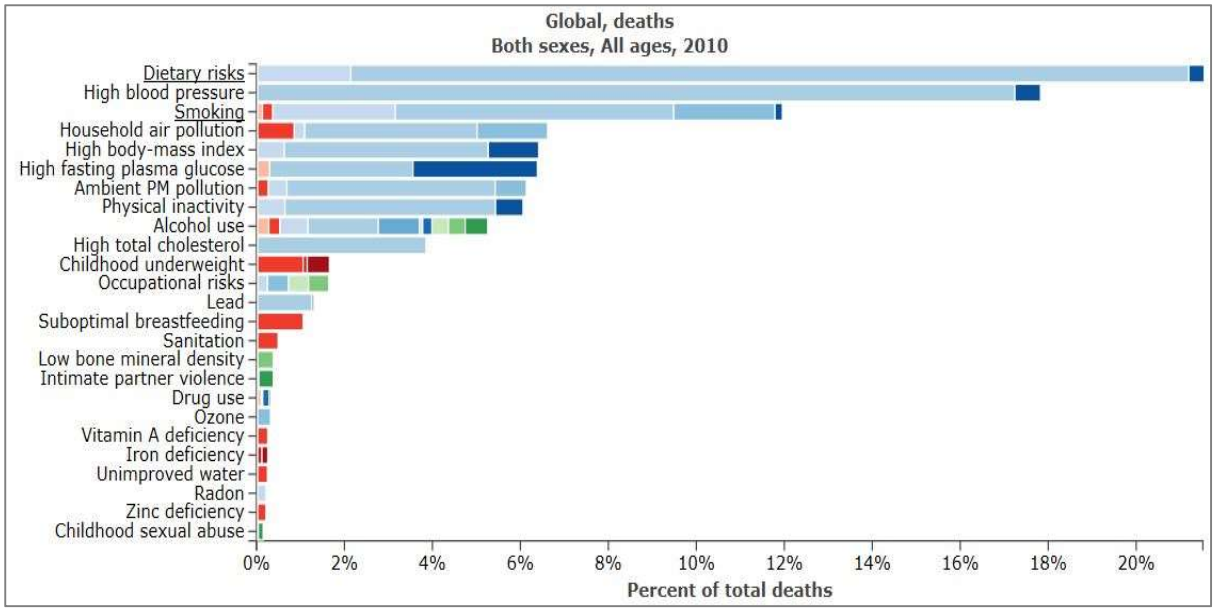

Figure 1.2. Smoking is responsible for ~12% (6.2 million) of all deaths globally (GBD, 2010).

1.2. The Vascular Endothelium and Endothelial Dysfunction

1.2.1. The Vascular Endothelium: Structure and Function

The endothelium is a thin, continuous layer of endothelial cells (ECs) that line the interior of all blood vessels and can be considered an active and dynamic tissue (Fig 1.3) (Gonzalez and Selwyn, 2003). ECs are flat with a large nucleus that often protrudes into the vascular lumen. The appearance of ECs varies according to their location, being spindle-shaped in arteries and arterioles and oriented in the direction of the blood flow, whereas in capillaries and venules ECs are of a more rounded shape (Mas, 2009). The endothelium consists of approximately ten trillion (10^{13}) cells and weighs almost one kg in an adult (Galley and Webster, 2004).

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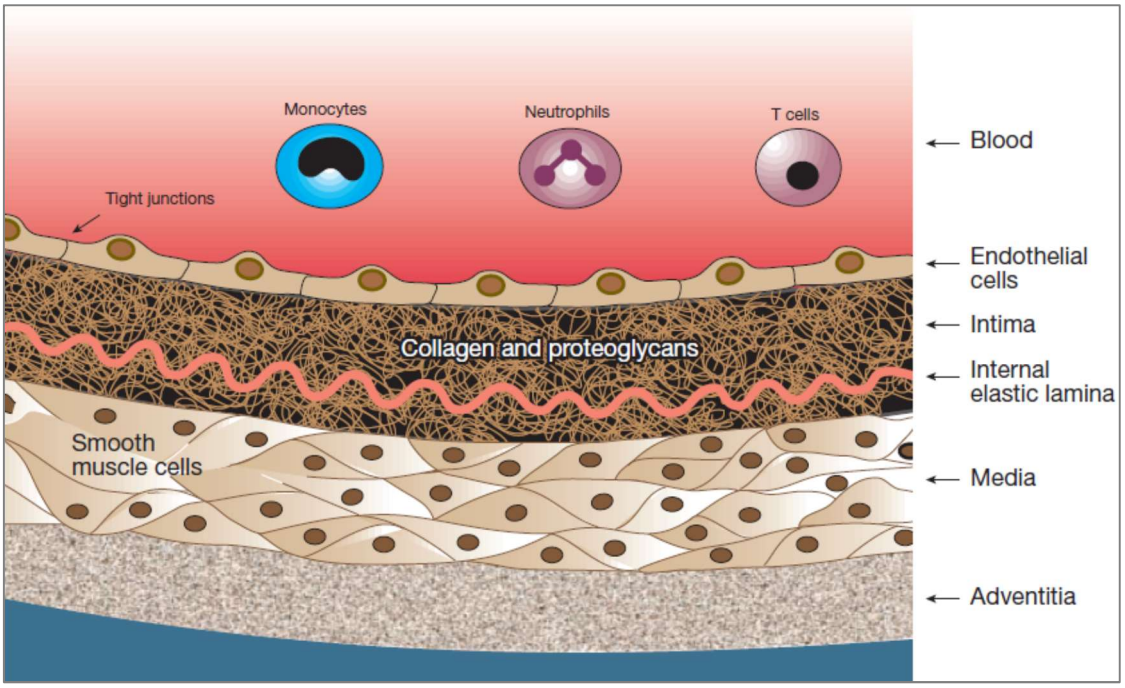

Figure 1.3. A normal large artery, consisting of three distinct layers; the intima, the media and adventitia. (Lusis, 2000)

ECs have a prominent Golgi apparatus, which suggests a significant secretory activity (Mas, 2009). Even though ECs have abundant mitochondria, most of their energy is supplied through anaerobic glycolysis, showing low oxygen consumption (Davidson and Duchen, 2007). ECs are attached to each other by zipper-like junctions along the cell borders and linked to networks of cytoskeletal and signalling proteins. Three types of junction structures have been identified: adherens junctions, tight junctions and gap junctions (Dejana, 2004). The luminal surface of the endothelium is covered with a negatively charged mesh-like structure made up of glycoproteins and proteoglycans, anchored to ECs by transmembrane protein domains. This structure is known as the endothelial glycocalix and has an estimated thickness of 50-100 nm (Rutledge *et al*., 2010) (Fig 1.4).

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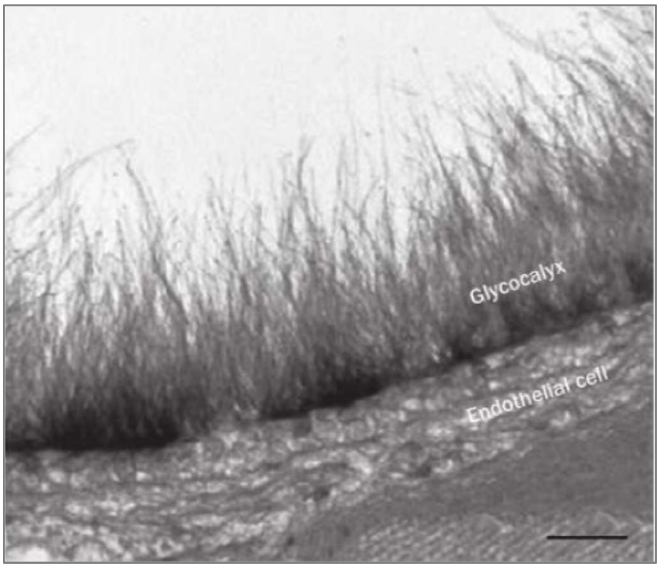

Figure 1.4. Electron microscopy image of the endothelial glycocalyx. Bar = 200 nm. (Rutledge et al, 2010)

The endothelium is involved in a number of important functions, such as maintenance of blood circulation and regulation of vascular tone, as well as synthesizing and releasing vasoactive substances, thus playing an important role in maintaining vascular homeostasis (Behrendt and Ganz, 2002).

1.2.2. Endothelium-Derived Vasoactive Factors

The endothelium maintains vascular homeostasis by releasing a variety of vasoactive factors. These factors can cause either dilation or constriction of the blood vessels, depending on the type of stimulus (Chhabra, 2009). Under normal circumstances vasodilator signals predominate compared to vasoconstrictor signals, but the delicate balance between these factors can be reversed by cardiovascular risk factors (Mas, 2009). Vasodilatory agents include nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarising factor (EDHF) (Table 1.1) (Mudau *et al*., 2012). Vasoactive factors responsible for vasoconstriction include endothelin-1 (ET-1), thromboxane A (TXA_2) and angiotensin II.

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Table 1.1. Overview of endothelium-derived vasoactive factors. (Mudau *et al*., 2012)

An Overview of Endothelium-derived Vasoactive Factors		
Endothelium-derived factors	**Physiological effects**	**Enzymatic source and mechanism of action**
Nitric Oxide (NO)	- Potent vasodilator - Inhibits inflammation, vascular smooth muscle cells (VSMCs) proliferation and migration, platelet aggregation and adhesion and leukocyte adhesion - Regulates myocardial contractility - Regulates cardiac metabolism - Cardio-protective during ischaemia-reperfusion injury	- Synthesized by the enzymes, eNOS, nNOS and iNOS, with eNOS being the major source of NO during physiological conditions - Diffuses from ECs to underlying VSMCs where it binds to soluble guanylyl cyclase (sGC), leading to a cascade of events that ultimately result in vascular relaxation
Prostacyclin (PGI_2)	- Vasodilatory agent - Inhibits platelet aggregation	- Derived from arachidonic acid by cyclooxygenase-2 (COX-2)
Endothelium-derived hyperpolarizing factor (EDHF)	- Exerts vasodilatory effects, particularly in small arteries of diameter ≤ 300 µm	- Its identity is still under suspicion with proposed candidates such as potassium ions and hydrogen peroxide
Endothelin-1 (ET-1)	- A potent vasoconstrictor	- Synthesized by endothelin-converting enzyme - Exerts its effects via two receptors: ET_A expressed on ECs and ET_8 on VSMCs. ET_A receptors promote vasoconstriction, whereas ET_8 receptors promote NO production and ultimately reduction in ET-1 production
Thromboxane A (TXA_2)	- A potent vasoconstrictor	- Derived from arachidonic acid by COX-1
Angiotensin II (Ang II)	- A potent vasoconstrictor	- Synthesized by angiotensin converting enzyme - Elicits its effects via two receptors: AT_1 which promotes vasoconstriction and cell proliferation, and AT_2 which antagonizes the effects of AT_1

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1.2.2.1. Nitric Oxide

It is only relatively recently that the role of NO as an important endogenous signalling molecule has come to the forefront (Neill *et al*., 2008). Until the discovery in 1987 that the previously unidentified molecule, only termed 'endothelium-derived relaxing factor' (EDRF) was in fact NO, the molecule was thought of merely as a toxic by-product of combustion (Ignarro *et al*., 1987). NO is a simple diatomic gas and free radical, properties that allow for easy passage between cells and tissues, while being able to react with a large number of molecules. These properties make NO an ideal signalling molecule, capable of a wide range of biological effects (Strijdom *et al*., 2009). NO is considered to be the most potent endogenously synthesized vasodilator in the body, and the pathophysiological consequences of reduced NO bioavailability is a key marker of endothelial dysfunction (Mudau *et al*., 2012).

NO is generated by nitric oxide synthases (NOS), of which three distinct isoforms exist, namely neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS) (Naseem, 2005). Both eNOS and nNOS are constitutive, calcium-dependent enzymes that continuously produce low levels of NO and studies in gene disrupted mice indicate that both nNOS and eNOS have anti-atherosclerotic actions (Liu and Huang, 2008). eNOS is constitutively expressed in the endothelium, following activation by acetylcholine or bradykinin, as well as by the shear stress of flowing blood (Li *et al*., 2014). However, iNOS is calcium independent and its expression provoked by inflammatory cytokines. This leads to the production of large amounts of NO, up to a 1000-fold more than eNOS or nNOS. The excess NO can react with the free radical superoxide anion ($O_2^{\bullet-}$) and yield highly reactive species, peroxynitrite (Strijdom *et al*., 2009). All NOSs require cofactors, such as (6R)-5,6,7,8-tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphyrin IX (haem) (Alderton *et al*., 2001).

It has been proposed that, of the three isoforms, eNOS is responsible for NO production under physiological conditions in the cardiovascular system, specifically in ECs. The production of NO by eNOS leads to the classical signalling mechanism in the underlying vascular smooth muscle cells (VSMCs) which, in turn, leads to relaxation (Dudzinski and Michel, 2007). Absence of some of the cofactors mentioned above or the failure of the eNOS protein to dimerise, will lead to the enzyme catalysing the formation of $O_2^{\bullet-}$ instead of NO, a mechanism referred to as eNOS uncoupling (Föstermann and Munzel, 2006).

It has been proposed that the release of endothelial derived NO can prevent leukocyte adhesion and subsequent migration into the subendothelial space (Kubes *et al*., 1991). NO can also down regulate gene expression of adhesive proteins and inhibit LDL oxidation by reacting with lipid peroxyl radicals (Rubbo *et al*., 1994). NO is responsible for protecting the

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vascular wall by inhibiting the actions of serotonin and thromboxane A_2, which are platelet-derived vasoconstrictive factors (Michel and Vanhoutte, 2010). In addition to supressing platelet aggregation, NO prevents endothelial cell apoptosis and inhibits VSMC proliferation and migration (Lei *et al*., 2013).

1.2.2.2. Endothelial Dysfunction

Endothelial dysfunction (ED) can occur when the balance between a vasodilatory and vasoconstrictory state is disrupted, resulting in ECs losing their ability to maintain homeostasis, ultimately leading to impairment of vasorelaxation, as well as increased adhesiveness of the endothelium for circulating inflammatory cells (Gonzalez and Selwyn, 2003). Even though the pathogenesis of ED is considered multifactorial, oxidative stress appears to be a common underlying cellular mechanism (Mudau *et al*., 2012). Oxidative stress can lead to the imbalance of endothelium-derived vasodilatory and vasoconstrictory factors, whereby the pro-vasoconstrictory states become dominant, leading to progressive pathophysiological changes. These endothelial changes can promote vascular adhesion, coagulation and proliferation and exhibit pro-inflammatory and pro-oxidant features (Chhabra, 2009). ED is an early precursor of atherosclerosis and is associated with a number of cardiovascular risk factors, including hypertension, diabetes and smoking (Cipollone *et al*., 2007). It has been shown that early endothelial changes, such as ED, are reversible (Hsueh *et al*., 2004) which has great clinical relevance in terms of early diagnosis, stratification and potential treatment of CVD. However, if risk factors are sustained and early endothelial changes not reversed progression to atherosclerosis and subsequent CVD will follow (Fig 1.5) (Mudau *et al*., 2012).

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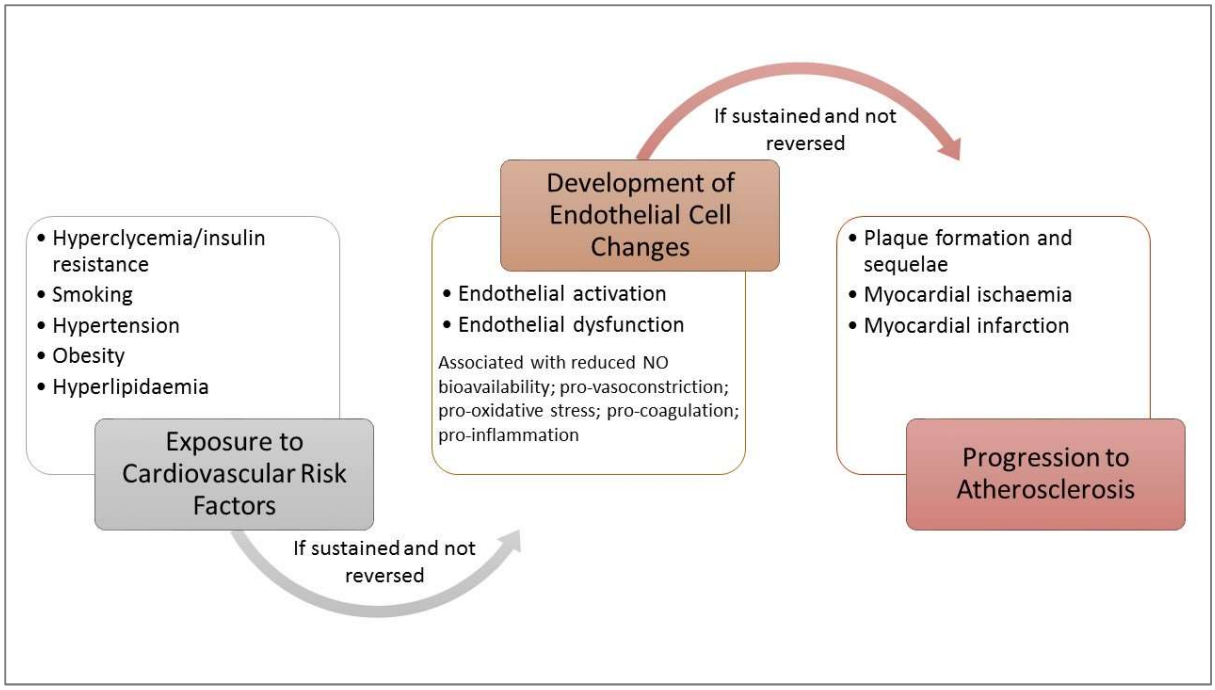

Figure 1.5. Exposure of endothelial cells to cardiovascular risk factors and the resultant pathophysiological changes. Adapted from Mudau *et al*., 2012.

1.3. Atherosclerosis

Atherosclerosis is a chronic inflammatory disease, which is characterised by the hardening and thickening of the blood vessels via the formation of an atherosclerotic plaque or lesion (atheroma), which can lead to vascular occlusion and limited blood flow. Atherosclerosis is associated with ischaemic heart disease, stroke and a number of other CVD conditions (Cipollone *et al*., 2007 and Fearon and Faux, 2009). ED is considered the first phase in the development of the atherosclerotic plaque and is followed by the inflammatory phase, the reparative phase and finally, the thrombotic phase (Naseem, 2005).

Cholesterol in the blood, specifically low-density lipoprotein-cholesterol (LDL-cholesterol) is able to passively diffuse into the arterial wall. LDL becomes trapped through the interaction between apolipoprotein B100 and matrix proteoglycans and is subject to oxidative modification due to prolonged exposure to reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidised LDL (OxLDL) is able to exert a pro-inflammatory response to produce chemokines, such as monocyte chemotactic protein-1, as well as growth factors. Adhesion molecules, such as P-selectin and vascular adhesion molecule-1 (VCAM-1) are also expressed. Through this process monocytes are recruited and their entry facilitated and differentiation into macrophages stimulated. As macrophages take up oxLDL through scavenger receptors, lipid laden foam cells are formed (Fig 1.6) (Lusis, 2000).

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The fibrous cap found on atherosclerotic plaques is a result of macrophages secreting growth factors and cytokines, which in turn promote migration and proliferation of VSMCs and secrete excess extracellular matrix proteins (Naseem, 2005). When foam cells die, a lipid-rich necrotic core, enclosed by a fibrous cap is left behind. These plaques can become more complex through calcification and haemorrhage, resulting from small blood vessels that grow into the lesion. The advanced plaques can grow large enough to block blood flow or rupture, leading to thrombosis, which usually results in myocardial infarction or stroke (Fig 1.7) (Lusis, 2000).

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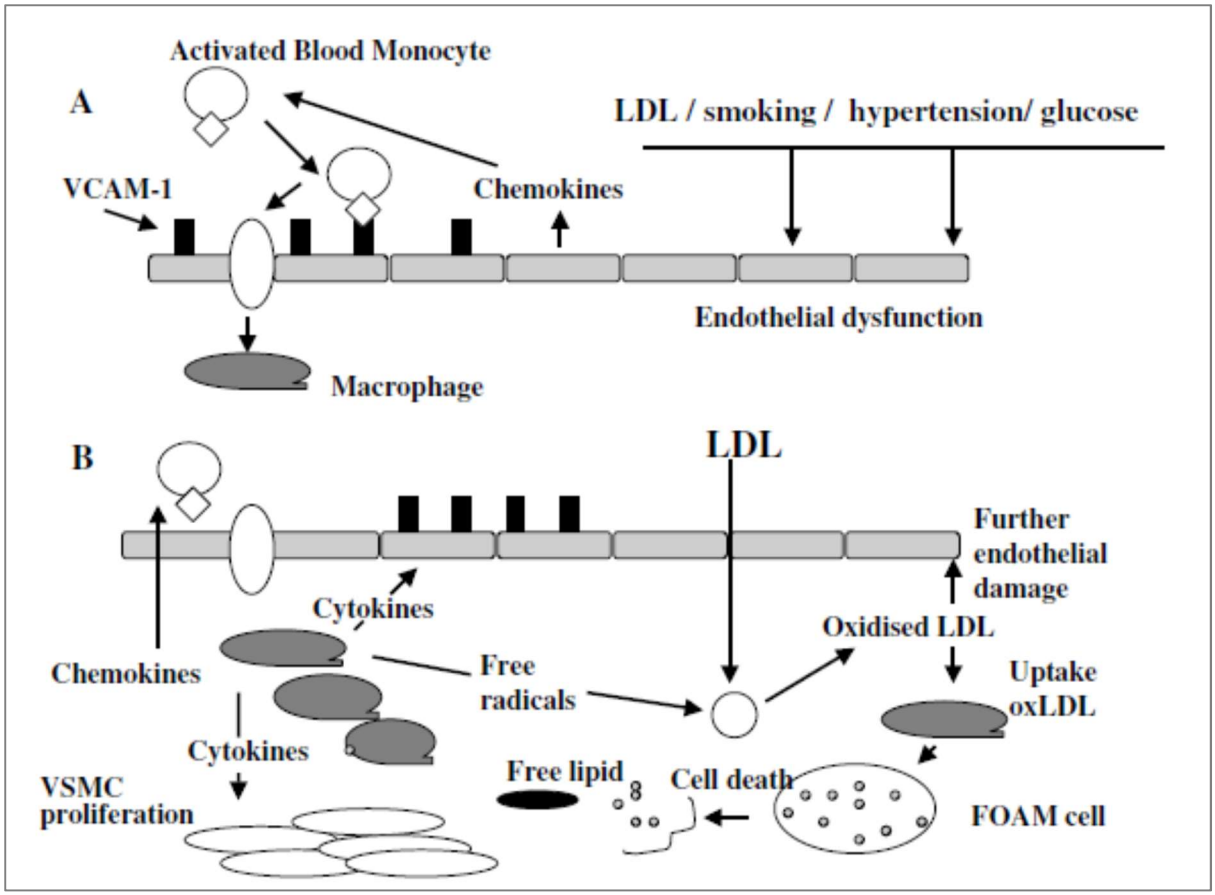

Figure 1.6. The first two phases in the development of the atherosclerotic plaque: (A) endothelial dysfunction; (B) inflammatory phase. (Naseem, 2005)

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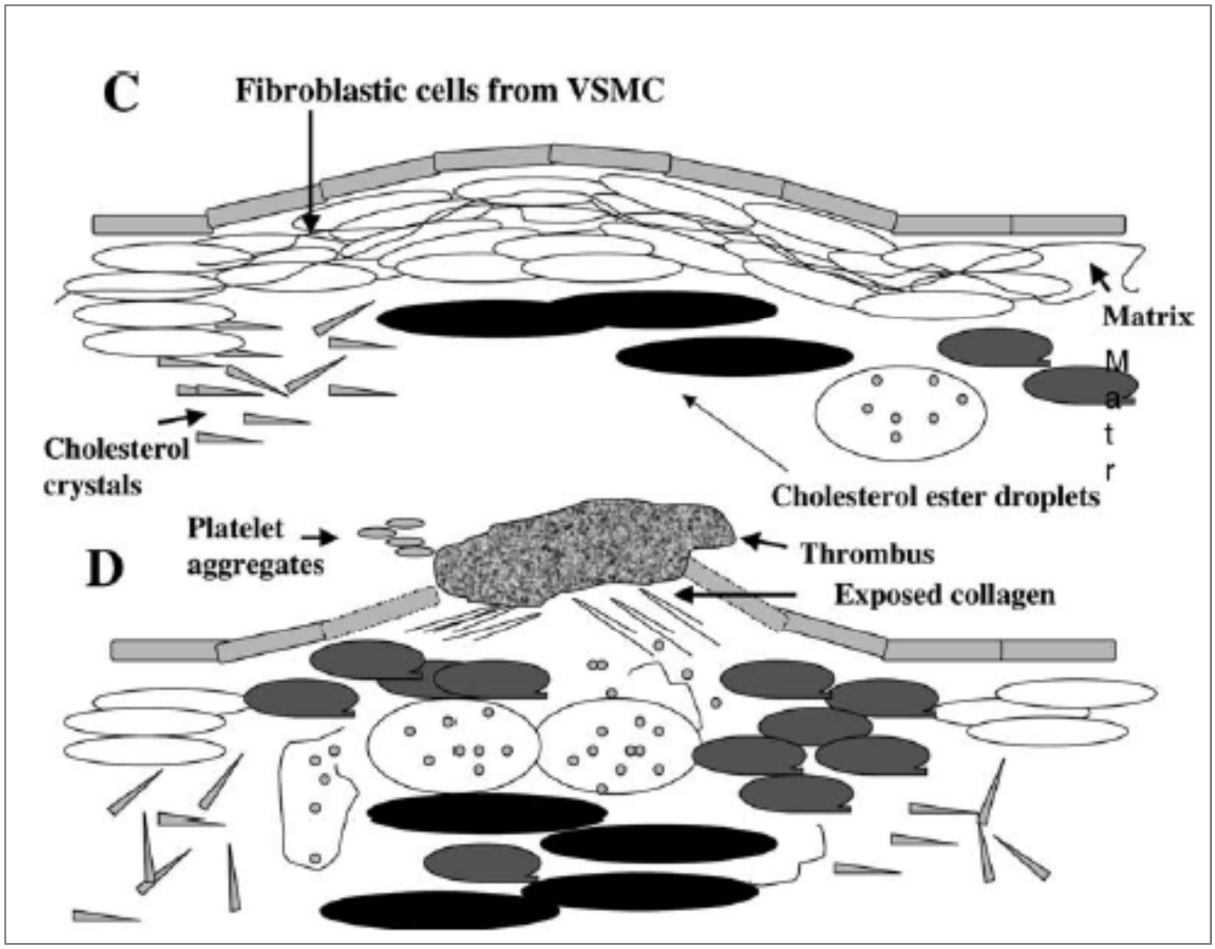

Figure 1.7. The advancement of atherosclerosis: (C) reparative fibrotic phase; (D) thrombotic phase. (Naseem, 2005)

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1.3.1. Vascular Oxidative Stress

The role of oxidative stress in the development of CVD has been recognized for many years, while studies indicate that altered oxygen utilization and an increase in ROS levels contribute to the progression of CVD (Wattanapitayakul and Bauer, 2001). The role of ROS in physiological signalling has been elucidated more recently and evidence indicates that signalling pathways modulated by ROS are complex and compartmentalized (Brown and Griendling, 2015). ROS reduce and eliminate the protective abilities of NO, which in turn will lead to endothelial dysfunction (Barua *et al*., 2003). Experimental and clinical data indicate that exposure to harmful substances such as nicotine increases oxidative stress and has the potential to induce endothelial dysfunction (Ambrose and Barua, 2004).

ROS include superoxide (O_2•-), hydrogen peroxide (H_2O_2), peroxynitrite (OONO-) and the hydroxyl radical (HO•), all of which are reactive molecules containing oxygen. ROS are constantly produced in biological systems and several endogenous defence mechanisms have been identified, including enzymes such as superoxide dismutase (SOD), which removes superoxide radicals by catalysing a dismutation reaction to hydrogen peroxide and oxygen (Ceconi *et al*., 2003) and catalase, which accelerates the decomposition of hydrogen peroxide to oxygen and water (Li *et al*., 2014). Glutathione peroxidase (GPx) is able to reduce hydrogen peroxide to water and lipid hydroperoxidases to alcohols (Lubos *et al*., 2011). Lipid peroxidation presents a further mechanism of cellular injury and is often used as an indicator of oxidative stress in cells (Yagi, 1998). Lipid peroxidases are derived from polyunsaturated fatty acids and are unstable. These unstable compounds decompose to form reactive carbonyl compounds such as malondialdehyde (MDA) (Wang *et al*., 2001). An increase in ROS as a result of various cardiovascular risk factors, such as hypertension, diabetes and smoking not only contributes to the development of atherosclerosis, but can also lead to a reduction in eNOS derived NO and its vasculature protective properties (Fig 1.8) (Li *et al*., 2014).

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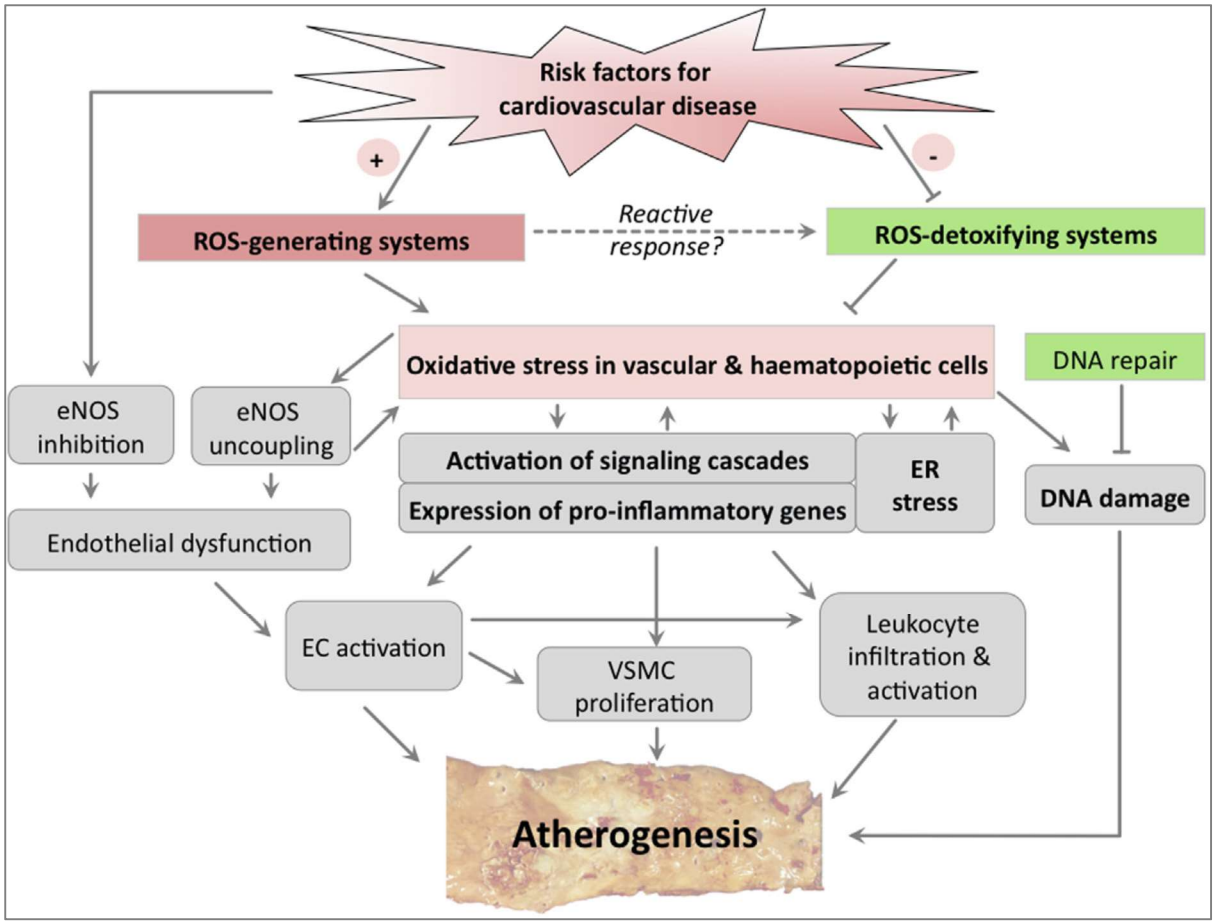

Figure 1.8. The role of oxidative stress in the progression of atherosclerosis, whereby ROS-producing enzymes are activated and/or ROS-detoxifying systems are affected by cardiovascular risk factors. (Li *et al*., 2014.)

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1.4. Nicotine

1.4.1. Nicotine Chemistry and Metabolism

Nicotine (3-[(2S)-1-Methyl-2-pyrrolidinyl]pyridine) is an alkaloid substance found in tobacco leaves where it naturally acts as a botanical insecticide (Fig 1.9). Nicotine comprises approximately 95% of the total alkaloid content of cigarette tobacco with about 1 – 1.5 mg of nicotine being absorbed systemically during smoking (Benowitz and Jacob, 1984). When smoking a cigarette, nicotine is distilled from tobacco and carried into the lungs by smoke particles and absorbed into the pulmonary venous circulation and then enters the arterial circulation. Peak arterial concentrations of nicotine can be reached within 20-30 seconds of inhalation. Nicotine then moves rapidly from the lungs to the brain, where it is able to bind to nicotinic acetylcholine receptors (nAChRs) (Rose *et al*., 1999).

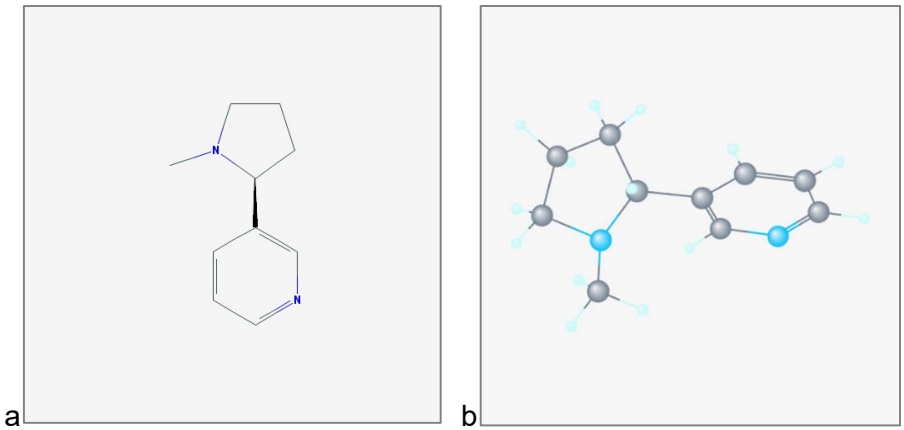

Figure 1.9. The 2D (a) and 3D (b) structure of nicotine.

nAChRs are ligand-gated ion channels present in both the peripheral nervous system (PNS) and the central nervous system (CNS). nAChRs respond to high and low doses of acetylcholine (ACh), as well as to systemically applied pharmacological agents, such as nicotine. Binding of nicotine results in the assembly of principal (α2, α3, α4, α6) and complementary (β2, β4) subunits, with the highly expressed α4β2 receptors displaying the highest level of upregulation (Nguyen *et al*., 2004). The activation of brain nAChRs result in the release of various neurotransmitters, including dopamine, serotonin, glutamate and GABA (γ-aminobutyric acid) (Taly *et al*., 2009). Nicotine primarily acts on the central nervous system by initiating action potentials in dopaminergic neurons when binding to nAChRs on the cell bodies in the ventral tegmental area (VTA). The release of dopamine gives nicotine its rewarding properties and is integral to its addictive nature (McRobbie and Thornley, 2008).

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Dopamine is released in the mesolimbic area, the corpus striatum and frontal cortex. Dopaminergic neurons in the VTA, as well as in the shell of the nucleus accumbens are critical in drug-induced reward and play a role in the perceptions of pleasure and reward (Fig 1.10) (Benowitz, 2010). After absorption and entering the bloodstream nicotine is distributed to body tissues, with a high affinity for the liver, kidney, spleen and lung. Nicotine binds to brain tissues with high affinity and, over time, the receptor binding capacity in smokers is increased, compared to non-smokers. This is caused by an increase in the number of nicotine cholinergic receptors in the brain of smokers (Perry *et al*., 1999). Nicotine is metabolised by the liver and six primary metabolites have been identified, the most important and abundant being a lactam derivative, cotinine (Benowitz, 2010).

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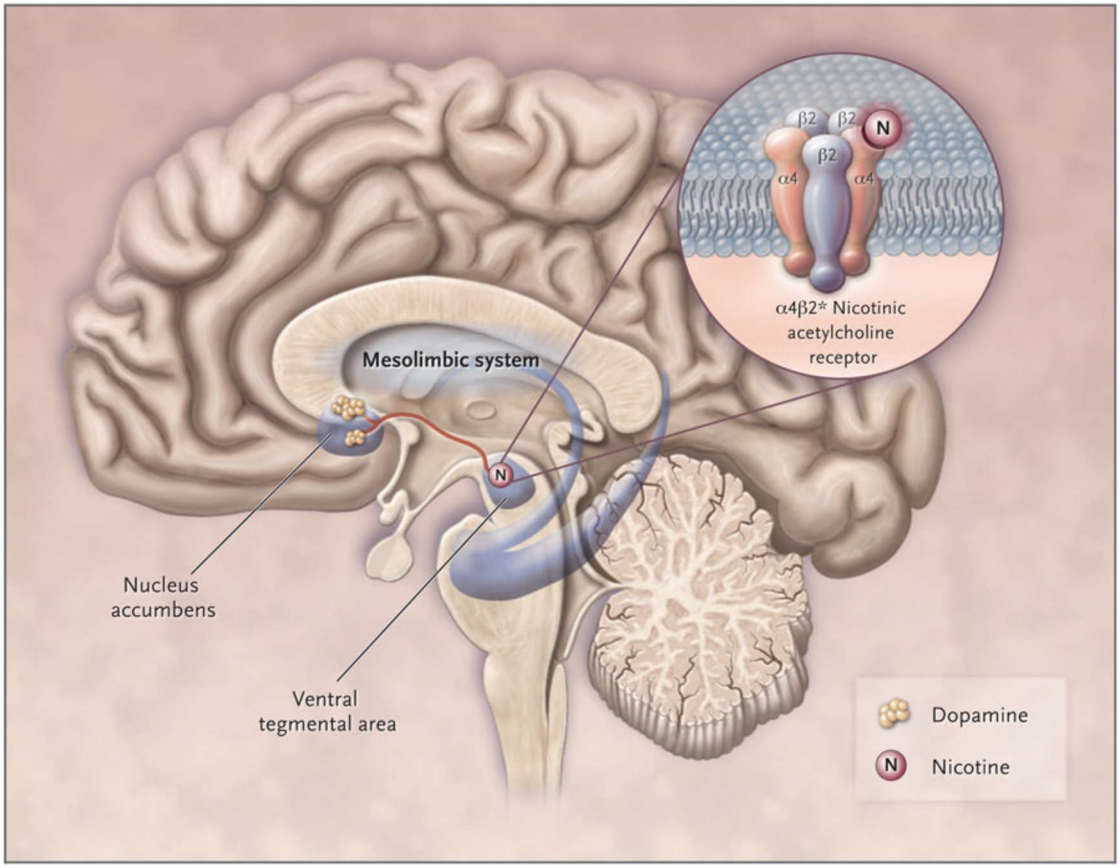

Figure 1.10. The role of the mesolimbic dopamine system in nicotine activity, whereby nicotine activates nACh receptors in the VTA. The result is a release of dopamine in the shell of the nucleus accumbens. Sketch taken from Benowitz, 2010.

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1.4.2. Nicotinic Acetylcholine Receptors (nAChRs) in the Vasculature

In addition to the nervous system, nAChRs are expressed ubiquitously in many cell types, including ECs and VSMCs (Lindstrom, 1997). The structure of vascular nAChRs closely resembles that of nAChRs in the CNS, namely a pentamer composed of distinct subunits, arranged tightly around a central pore (Egleton *et al*., 2009). In humans, 16 isoforms of subunits have been identified, including α subunits (α_1-α_{10}), β subunits (β_1-β_4), γ, δ and ε subunits (Gotti *et al*., 2007). While ACh is the endogenous ligand for nAChRs, nicotine has also been shown to be a high-affinity vascular nAChR ligand. Ligand binding leads to conformational changes in the nAChR, resulting in opening of the ion channel and transmission of signals (Santanam *et al*., 2012). Binding of nicotine to vascular nAChRs are capable of exerting direct action on multiple cell types in the cardiovascular system. Cell types include VSMCs, endothelial cells, platelets and other cellular effectors of importance in the formation of the atherosclerotic plaque (Egleton *et al*., 2009). It has also been shown that several components of atherogenesis, including inflammation, proliferation and migration are regulated through non-neural nicotinic cholinergic pathways (Bauwens *et al*., 2015). Nicotine stimulates nAChR mediated VSMC migration via important signalling pathways, including increased calcium influx, activation of epidermal growth factor receptor (EGFR) and phosphorylation of Ets-like gene 1 (Kanda and Watanabe, 2007; Vazquez-Padron *et al*., 2010).

The α7 nAChR subunit is of particular interest in the development of atherosclerosis, since it is found in non-neural cells, such as macrophages (Wang *et al*., 2003) and has also been detected in rat arterial systems (Bruggmann *et al*., 2002). It has been demonstrated that, while ECs express most of the known mammalian nAChR subunits, the effects of nicotine are largely mediated through the α7 nAChR, while the other subunits play a modulatory role (Wu *et al*., 2009). Furthermore, studies have shown that the α7 nAChR plays a critical role in EC proliferation, survival, migration and tube formation (Heeshen *et al*., 2002). These findings are further substantiated by studies indicating that α-bungarotoxin, a selective antagonist of homomeric nAChRs and the neuromuscular nAChR, can inhibit nicotine-induced EC angiogenic processes (Ng *et al*., 2007). These mechanisms mediated by nAChRs, specifically α7 nAChR, may contribute to the pathological neovascularization associated with nicotine use, and could have therapeutic relevance (Wu *et al*., 2009).

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1.4.3. Nicotine and Atherosclerosis

Nicotine is associated with the development of several diseases, including CVD, emphysema and cancer, all of which present abnormal neovascularization as a common feature (Costa and Soares, 2009). Nicotine exerts significant cardiovascular effects, such as an increase in heart rate and myocardial contractility, as well as an increase in myocardial uptake and oxidation of free fatty acids. A summary of the cardiovascular effects of nicotine are given in Table 1.2 (Kilaru *et al*., 2001). It has been shown that coronary blood flow has a biphasic response to nicotine. Initially nicotine intake increases blood flow in the large coronary vessels and smaller resistance vessels, after which there is a decrease in blood flow. The decrease in blood flow is mediated by alpha-adrenergic vasoconstriction of coronary resistance vessels (Kaijser and Berglund, 1985). Nicotine increases blood pressure and may contribute to the acceleration of hypertension by its pro-vasoconstriction effects, as well as promoting interactions between platelets and the endothelial cell lining (Downey *et al*., 1981). It has also been shown that nicotine is able to affect lipid metabolism by increasing LDL-cholesterol and decreasing high-density lipoprotein (HDL) cholesterol, while resulting in higher levels of OxLDL (Clutte-Brown *et al*., 1986; Harats *et al*., 1989). Nicotine users exhibit decreased NO bioavailability and impaired flow-mediated vasodilation, characteristics which are highly suggestive of ED (Puranik and Celermajer, 2003).

Table 1.2. Cardiovascular effects of nicotine (Kilaru *et al*., 2001).

Increase in heart rate and myocardial contractility
Increase in cardiac output, stroke volume, and coronary blood flow
Cutaneous vasoconstriction
Subcutaneous vasoconstriction
Coronary vasoconstriction
Increase in muscle blood flow
Hyperlipidaemia: increased LDL-cholesterol, decreased HDL-cholesterol
Endothelial injury
Platelet activation and thrombosis
Increased levels of fibrinogen
Increased circulating catecholamines
Intimal hyperplasia

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Nicotine has been shown to be a pro-angiogenic factor, whereby new blood vessels can develop from pre-existing ones. Angiogenesis is a dynamic process regulated by the balance between angiogenic activating and inhibiting factors (Costa *et al*., 2007). Angiogenesis relies on various changes to the matured vessel, including pericyte detachment, extracellular matrix (ECM) degradation and remodelling, followed by proliferation, migration and assembly of ECs into tubule structures (Costa *et al*., 2007). The proliferation, migration and adhesion of SMCs are also associated with angiogenesis (Fam *et al*., 2003). While angiogenesis plays an important role in many physiological situations, such as ovulation, embryogenesis and placental development, excess angiogenesis can lead to a variety of pathological situations, such as atherosclerotic plaque development, diabetic retinopathy and cancer (Pandya *et al*., 2006). Genetic predisposition has also been suggested to influence the development of atherogenesis in individuals exposed to nicotine, however the importance of genetic variants remain unknown. It has been proposed that either CYP1A1 MSP polymorphism or certain eNOS intron 4 polymorphisms increase susceptibility to nicotine exposure related atherosclerosis development (Wang *et al*., 2002).

Inflammation associated with nicotine intake has been suggested to be an essential component in the initiation and evolution of atherosclerosis. Cigarette smoking causes an increased level of multiple inflammatory markers, including C-reactive protein (CRP), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (Ambrose and Barua, 2004) and nicotine has been shown to significantly affect CRP, IL-6 and TNF-α levels in newborn rats after prenatal nicotine-exposure (Mohsenzadeh *et al*., 2014). Interest in the relevance of these circulating inflammatory biomarkers to coronary heart disease has increased greatly since the link between inflammation and atherosclerosis became known (Danesh *et al*., 2008).

CRP, a phylogenetically highly conserved plasma protein, is a liver-derived inflammatory biomarker which participates in the systemic response to inflammation and is considered part of the acute phase inflammatory response (Black *et al*., 2004). CRP induction is primarily regulated at the transcriptional level by IL-6, the effect of which can be enhanced by interleukin-1β (IL-1β) (Kushner *et al*., 1995). IL-6 has been shown to play a central part in the downstream inflammatory response responsible for atherosclerosis and is essential in the initiation and progression of the atherosclerotic lesion (Hartman and Frishman, 2014). According to various worldwide studies, CRP predicts future risk for CVD in apparently healthy individuals, including myocardial infarction, coronary artery disease, stroke and peripheral arterial disease (Ridker, 2003). In addition, the presence of CRP mRNA and proteins have been demonstrated in human atherosclerotic lesions and vascular cells (Calabro *et al*., 2003; Dong and Wright, 1996), futher underscoring the importance of CRP as an inflammatory biomarker for atherosclerosis.

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TNF-α is considered one of the most important inflammatory cytokines, playing a crucial role in innate and adaptive immunity, cell proliferation and apoptotic processes (Pober *et al*., 2006). There is evidence to suggest that TNF-α impairs endothelium-dependent and NO-mediated vasodilation in vascular beds (Gao *et al*., 2007; Picchi *et al*., 2006), possibly via down regulation of eNOS activation and expression, thereby reducing or diminishing NO production (Goodwin *et al*., 2007; Picchi *et al*., 2006). The involvement of TNF-α in the induction of ED and the initiation of atherosclerosis is further supported by the beneficial effects of anti-TNF-α agents on endothelial function in patients with chronic inflammatory diseases (Cardillo *et al*., 2006; Bilsborough *et al*., 2006). Elevation of these inflammatory cytokines in turn leads to the local recruitment of leukocytes on the surface of ECs, which is an early event in atherosclerosis (Bermudez *et al*., 2002). CRP, IL-6 and TNF-α have recently been used to demonstrate the association between smoking quantity and inflammation, leading to hypertension (Feng *et al*., 2013). As such, CRP, IL-6 and TNF-α are useful markers of inflammation associated with nicotine exposure and the initiation and progression of atherosclerosis.

1.4.4. Nicotine Addiction and Therapy

The potentially harmful effects of tobacco use has only been proven reasonably recently. However, the, captivating influence of tobacco was known by the Native Americans and this concept quickly spread to the Europeans. Francis Bacon, the English philosopher wrote the following on the spread of tobacco:

> ‘In our time the use of tobacco is growing greatly and conquers men with a certain secret pleasure, so that those who have once become accustomed thereto can later hardly be restrained therefrom.’ (Francis Bacon, *Historia vitae et mortis*, 1623)

The truly addictive nature of nicotine has only been accepted by the scientific community reasonably recently, since tobacco smoking was originally seen as merely a habit (Russell, 1971). The importance of deciphering the mechanisms of nicotine addiction became more important as the link between tobacco smoking and certain diseases, such as lung cancer, became more evident (Dani and Balfour, 2011). Nicotine addiction is extremely complex and smokers rely on cigarettes to induce pleasure, reduce stress and improve concentration, while relief from withdrawal symptoms directly enhance mood and performance. Withdrawal symptoms can be severe and include depressed mood and anxiety, as well as anhedonia (feeling that there is little pleasure in life) can also occur (Hughes, 2006).

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By the 1980s, the nicotine dependence hypothesis and the withdrawal effects associated with smoking cessation had been accepted. Along with this the importance of using nicotine replacement therapy (NRT) to aid cessation became prominent (Dani and Balfour, 2011). The most popular forms of NRT include transdermal nicotine patches and nicotine gum, while the recent invention and popularization of electronic cigarettes (e-cigarettes) provide a further possibility for such treatments. While it seems unlikely that nicotine, which is directly implicated in the onset and acceleration of atherosclerosis and, in turn, CVD should be used to assist with smoking cessation, it remains popular and effective (Elam, 2015). It is important to note that cigarette smoke is a complex mixture of substances, some of which are potentially cardiotoxic, such as carbon monoxide. In addition to eliminating carbon monoxide, a hypercoagulable and thrombosis promoting state is also not found when NRT is used, possibly due to lower levels of circulating epinephrine. Increased heart rate and blood pressure, which in turn increases myocardial work is also reduced with the use of NRT (Benowitz and Gourlay, 1997).

Studies examining the effect of NRT on cardiovascular parameters have produced conflicting results (Vansickel *et al*., 2010; Farsalinos *et al*., 2014). Some studies have shown increases in heart rate and blood pressure following NRT use (Fishbein *et al*., 2010; Yugar-Toledo *et al*., 2005; Najem *et al*., 2006), while other studies showed no effect (Blann *et al*., 1997; Tanus-Santos *et al*., 2001) on these parameters. It has been suggested that these discordant findings could be due to smokers, particularly heavy smokers, developing tolerance to the pressor effects of nicotine, resulting in hemodynamic markers being unaffected (Tanus-Santos *et al*., 2000). Interest in the safety of NRT in patients, specifically with those CVD, has led to the development of large clinical trials. In a five week, randomized, double-blind trial patients with coronary disease who smoked at least one pack per day received either nicotine patches or placebo in addition to weekly counselling sessions. Despite there being no differences in the frequencies of angina or arrhythmias between the groups, the use of NRT led to more frequent cessation of smoking in these groups (Working Group for the Study of Transdermal Nicotine in Patients with Coronary Artery Disease, 1994). Similar trends have been seen in recent studies, where nicotine patch users and non-users showed no difference in 7-day, 30-day or 1-year mortality after unstable angina (Meine *et al*., 2005). In a database study of 663 smokers, following the first year after an acute coronary event, no association was found between NRT and risk of adverse cardiovascular events (Woolf *et al*., 2012).

Based on pharmacologic mechanisms, NRT could cause deleterious cardiovascular effects, however, clinical studies are as yet, inconclusive and have been suggested to be underpowered to sufficiently demonstrate the potential risks. Despite inconclusive evidence of the safety of NRT, these treatments are associated with improved smoking cessation rates,

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making them clinically appropriate to use in patients who wish to quit smoking (Sobieraj *et al*., 2013). Furthermore, it has been shown that consultations with a physician as well as participation in support groups and counselling sessions are extremely important if smoking cessation is to be successfully achieved (World Health Organization, 2015). Until clinical studies yield more conclusive results as to the potential deleterious cardiovascular effects of NRT, it is worth considering the possible harmful effects of these treatments, including measures to modulate deleterious cardiovascular effects.

1.5. Rooibos (*Aspalathus linearis*)

Rooibos (*Aspalathus linearis*) is an indigenous South African herb, as well as a popular beverage and has long been known to possess biological properties (Fig 1.11) (Robak and Gryglewski, 1996). Rooibos has potent antioxidant, immune-modulating and chemo-protective actions, with the additional benefit of causing no adverse effects (McKay and Blumberg, 2007). In addition, it has been established that even though both unfermented and fermented rooibos are able to increase plasma antioxidant defences, unfermented rooibos has a higher antioxidant potency than fermented rooibos tea (Villaño *et al*., 2010). In addition, it has been shown that consumption of fermented rooibos can improve the lipid profile and redox status, which is relevant in the development of CVD (Marnewick *et al*., 2011).

a  b 

Figure 1.11. a) Rooibos growing on a farm in the Clanwilliam district of South Africa and b) dried rooibos leaves and tea bags. Images obtained from the internet. Websites: a) www.thedailytea.com and b) www.lauradutoit.hubpages.com.

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1.5.1. Chemical Composition and Colour Formation of the Fermentation Process

Health claims pertaining to the beneficial effects of rooibos are mostly based on the high polyphenol content of the species. Rooibos boasts a unique flavonoid content and contains various dihydrochalcones, including aspalathin, a C-linked dihydrochalcone glucoside (Koeppen and Roux 1965, 1966, Rabe *et al*., 1994) and aspalalinin, a cyclic dihydrochalcone (Shimamura *et al*., 2006), which are both unique to *Aspalathus linearis* (Fig 1.12). The 3-dehydroxy dihydrochalcone, nothofagin, is also present, this rare compound has previously been shown to be present only in the heartwood of *Nothofagus fusca* (Hillis and Inoue, 1967).

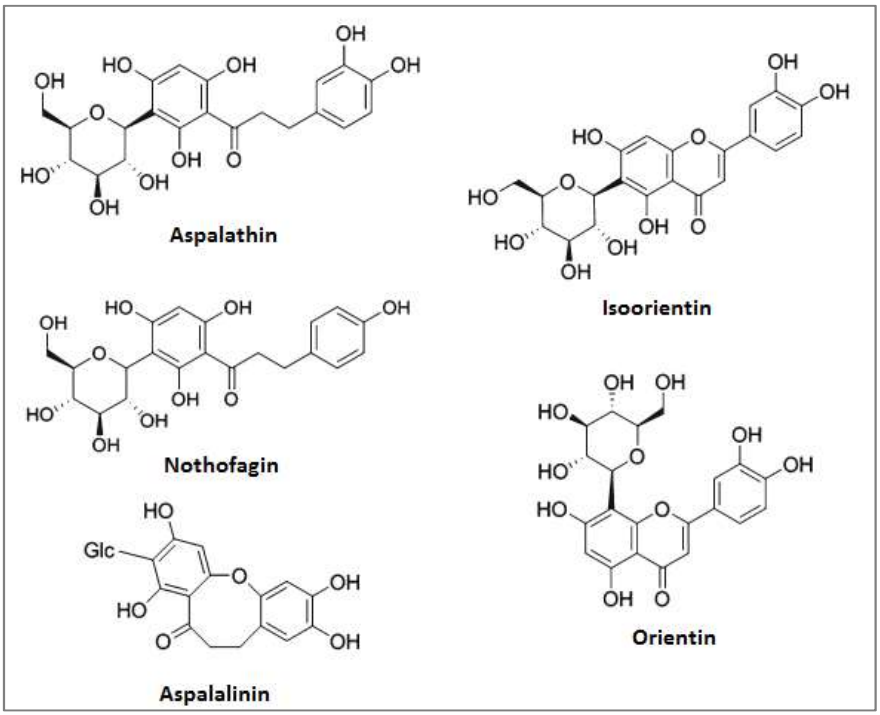

Figure 1.12. Chemical structure of specific flavonoids present in rooibos, including the unique aspalathin and aspalalinin. (Joubert *et al*., 2008)

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The degradation of green rooibos to the characteristic red-brown fermented product is a result of the non-enzymatic oxidative degradation of aspalathin (Krafczyk *et al*., 2009). It has been shown that the aspalathin content of rooibos can be reduced by as much as 98% during the fermentation process (Schulz *et al*., 2003). Two mechanisms of degradation of aspalathin have been elucidated to date. The first mechanism entails the oxidation of aspalathin to o-quinone, which is then converted to isoorientin and and orientin via eriodictyol glucopyranosides (Krafczyk and Glomb, 2008). Secondly, oxidation can initiate the dimerization of aspalathin. During this process the o-quinone is attacked nucleophylically by a second aspalathin molecule (Krafczyk *et al*., 2009).

In addition to two colourless dimers formed during initial oxidation (as described by Krafczyk *et al* in 2009), two further colourless substances are formed during continued oxidation, but later completely degraded. Two coloured structures with dibenzofuran skeletons have also been identified and their importance as key chromophores in the chemistry of colour formation during fermentation demonstrated (Heinrich *et al*., 2012).

1.5.2. Potential Health Benefits of Rooibos

For a long period of time the health benefits of rooibos were merely anecdotal. However, *in vitro* and *in vivo* studies have shown the potential treatment value of rooibos. Both unfermented and fermented rooibos have been shown to increase plasma total antioxidant capacity in humans, thus underlying its potential modulating effects, even though it has been suggested that fermentation can reduce the protective effect of rooibos (Villaño *et al*., 2010 and Marnewick *et al*., 2009).

Rooibos is commonly used for treating diarrhoea and abdominal spasm. The antispasmodic effects of rooibos have been demonstrated in isolated rabbit jejunum preparations. It has been proposed that the antispasmodic effects of rooibos is mediated mainly through K^+-channel activation (Gilani *et al*., 2006).

Since obesity and related metabolic diseases are on the increase globally (Guh *et al*., 2009), rooibos has also attracted attention as a potential safe and non-caloric intervention (Sanderson *et al*., 2014). Studies in mice receiving an increased energy intake Western-type diet and aqueous rooibos extract intervention lead to significant reductions in serum cholesterol, triglyceride and free fatty acid concentrations. The study suggested that the activation of AMP-activated protein kinase (AMPK), resulting in the regulation of energy homeostasis played a role in the effects of rooibos (Beltrán-Debón *et al*., 2011).

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The cardioprotective properties of rooibos have also been demonstrated. A six week trial in 40 human volunteers, consuming six cups of fermented rooibos per day, resulted in a significantly improved lipid profile and redox status, when compared to controls, which is relevant to heart disease in adults at risk for developing CVD (Marnewick *et al*., 2011). This is specifically relevant, since fermented rooibos is the traditional form in which the beverage is consumed. A seven week treatment with rooibos offered cardioprotection against ischaemia/reperfusion in isolated perfused rat hearts. Protection was attributed to the high flavonoid content of rooibos, resulting in a significantly decreased level of cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP), which are both pro-apoptotic proteins, as well as an improved GSH/GSSG ratio (Pantsi *et al*., 2011). The cardioprotective effect of fermented rooibos has also been demonstrated on cultured cardiomyocytes derived from diabetic rats, whereby pretreatment with rooibos provided better protection than the known antioxidant vitamin E in a H_2O_2 or ischaemic injury model (Dludla *et al*., 2014). A dose-dependent fall in arterial blood pressure has been shown in anaesthetized rats, where blood pressure was measured through carotid artery cannulation (Khan and Gilani, 2006).

However, despite the promising results generated in the past few years, studies into the cardioprotective effects of rooibos, both fermented and unfermented, remain limited, with investigations into the effects of rooibos in the vascular endothelium lacking in particular.

1.6. Melatonin

1.6.1. Melatonin Synthesis, Secretion and Binding

The hormone melatonin (N-acetyl-5-methoxytrypamine), a derivative of the essential amino acid tryptophan, was first isolated from the bovine pineal gland by Lerner *et al* (1958). Melatonin is a small molecule with highly lipophilic and hydrophilic properties, enabling it to cross biological barriers (Venegas *et al*., 2012) and is present in all bodily fluids, tissues and cellular compartments (Reiter *et al*., 2013). The suprachiasmatic nucleus (SCN) is the central circadian pacemaker and controls the production of melatonin by triggering the pineal gland to produce melatonin at night (Fig 1.13). The duration of release of melatonin is proportional to the daily dark period, whereby the period of melatonin release is termed ‘biological night’ (Arendt and Skene, 2005). The synthesis of melatonin occurs through two enzymatic steps. Serotonin is firstly acetylated by serotonin N-acetyltransferase [arylalkylamine N-acetyltransferase (AA-NAT)] to N-acetylserotonin. Secondly, a methyl group from (S)-adenosylmethionine is transferred to the 5-hydroxyindole-O-methyl-transferase (HIOMT) (Fig 1.14) (Klein, 2007).

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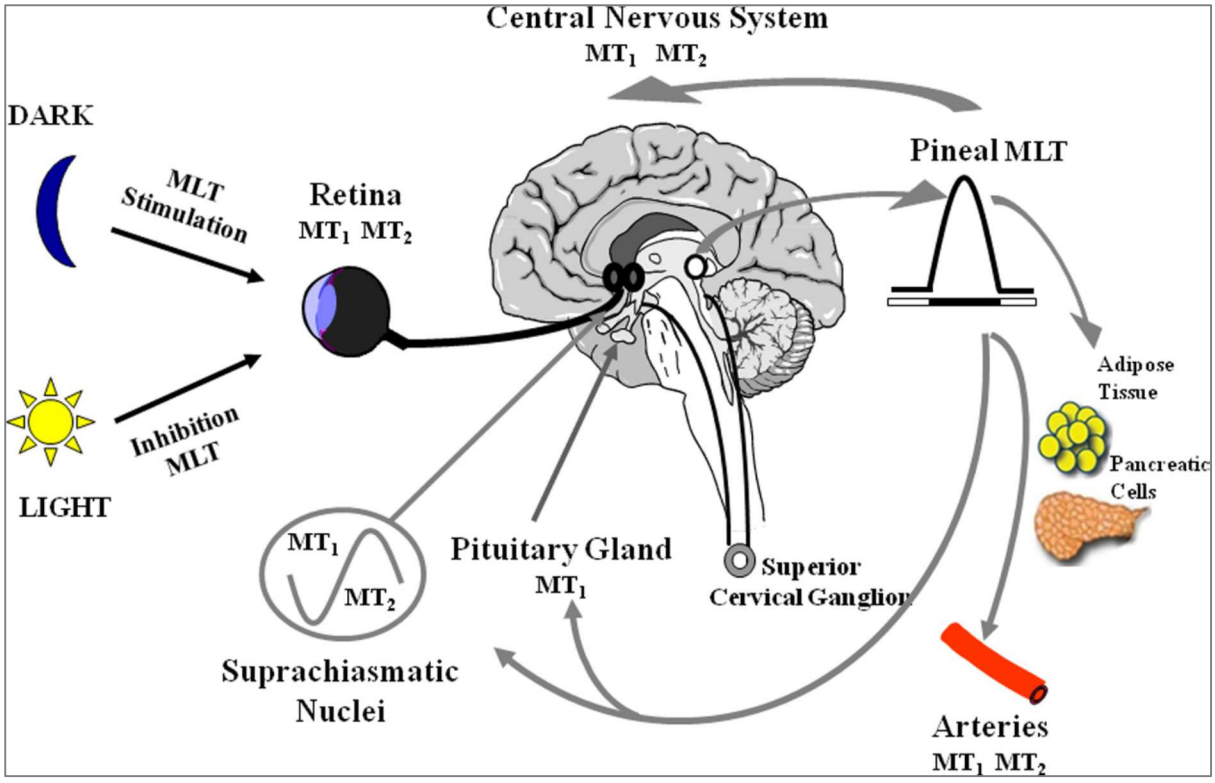

Figure 1.13. Synthesis and regulation of melatonin. The synthesis of melatonin by the pineal gland is controlled by the SCN, while G protein-coupled proteins, MT1 and MT2, respond to melatonin levels. (Dubocovich *et al*., 2010)

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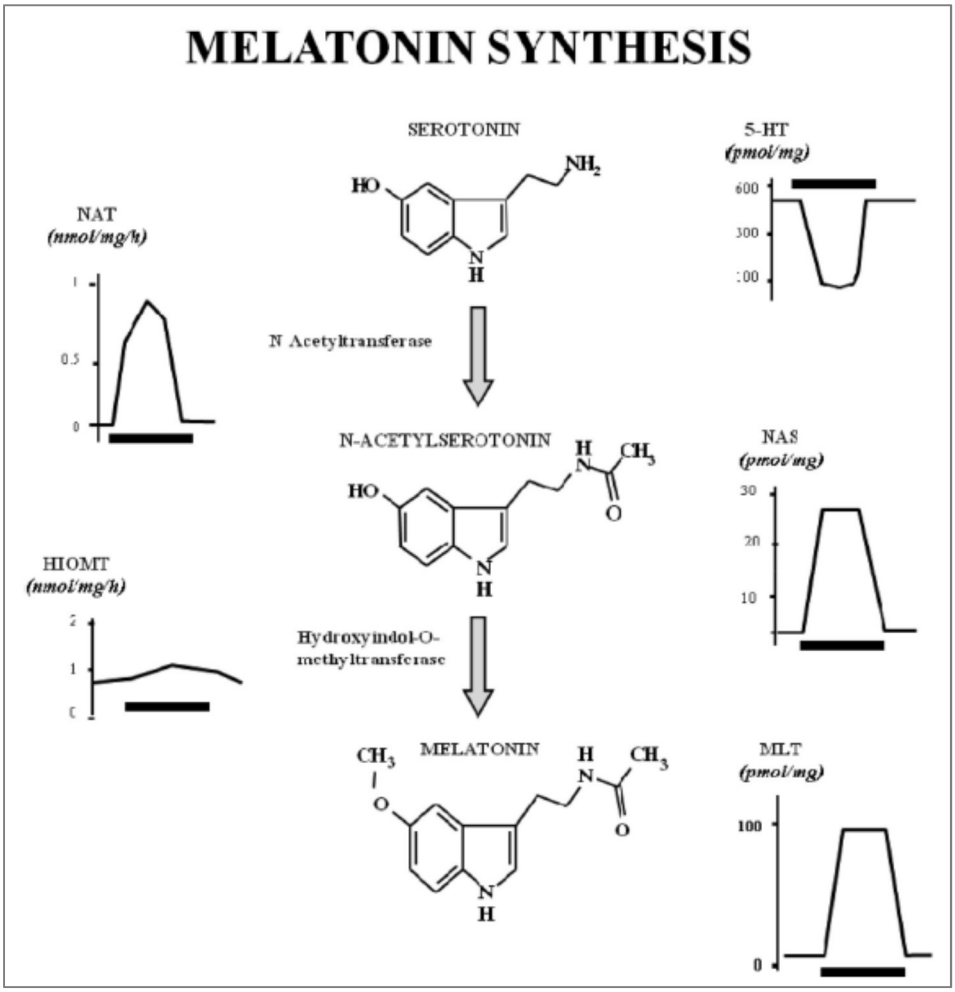

Figure 1.14. Melatonin synthesis. Melatonin is synthesized from serotonin through two enzymatic steps. Firstly, serotonin is acetylated AA-NAT to N-acetylserotonin, after which a methyl group from (S)-adenosylmethionine is transferred to the HIOMT. (Klein, 1999)

Secretion of melatonin peaks during the daily dark phase (usually six to eight hours after the onset of darkness), but is very low during the day (light phase) (Dominguez-Rodriguez *et al*., 2009; Reiter 1993). Melatonin receptors, located in target tissues, respond to melatonin levels, according to the circadian rhythm, which allows for high melatonin levels at night. These G protein-coupled receptors, termed MT1 and MT2 are also potential therapeutic targets for a variety of disorders, ranging from insomnia and depression to cardiovascular diseases and cancer (Dubocovich *et al*., 2010). Since melatonin has a limited duration of action, with a half-life of 0.54-0.67 hours in the blood, the synthesis of an analogue with a longer duration of action will be beneficial if used for its therapeutic value (Oxenkrug and Summergrad, 2010).

Originally, melatonin was viewed only to have endocrine function, but even though it performs mainly hormonal functions, modern endocrinology has shown that certain hormones, including

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melatonin exhibit additional actions and are therefore not strict classical hormones (Tan *et al*., 2003). In addition to the pineal gland, melatonin is secreted in a variety of organs (regarded as non-endocrine organs) and tissues, including the retina, Harder's glands, gastroenteric mucous membrane, megakaryocytes, platelets, lymphocytes, bone marrow and skin, but at lower and varying rates (Claustrat *et al*., 2005, Slominski *et al*., 2012). Earlier reports indicate that as much as 20% of circulating melatonin is not derived from the pineal gland (Gern and Norris, 1979).

In humans melatonin is generally converted to 6-hydroxymelatonin by the liver, clearing up to 97% of circulating melatonin in a single pass (Young *et al*., 1985). The 6-hydroxymelatonin is conjugated and excreted into urine as a sulphate derivative and glucuronide to a lesser extent (Ma *et al*., 2008).

1.6.2. Potential Health Benefits of Melatonin

In addition to controlling circadian rhythms, melatonin has recently been shown to be a versatile biological signalling molecule (Pandi-Perumal *et al*., 2006), involved in many other physiological processes in humans and animals, including blood pressure control, regulation of the immune response and free radical scavenging, thus indicating its importance throughout the body (Hardeland *et al*., 2006, Rodella *et al*., 2013). Under experimental conditions, chronic melatonin administration was demonstrated to be cardioprotective, which can be attributed to its free radical scavenging and antioxidant properties (Lochner *et al*., 2006, Nduhirabandi *et al*., 2011). While other naturally-occurring antioxidants, such as vitamins E and C and glutathione are known to reduce oxidative damage at different sites and to varying degrees, melatonin seems to have an even greater efficacy, possibly due to its wide spread intracellular distribution throughout the body (Favero *et al*., 2014).

Originally, melatonin was shown to detoxify the highly toxic hydroxyl radical (HO•) (Tan *et al*., 1993). It has since been shown that melatonin has the capability to scavenge hydrogen peroxide (H_2O_2) (Tan *et al*., 2000), hypochlorous acid (HOCl) (Zavodnik *et al*., 2004), superoxide anion radical (O_2•-) (Ximenes *et al*., 2005) and peroxynitrite anion ($ONOO^-$) (Reiter *et al*., 2001). Melatonin and its metabolites are able to neutralize oxygen derivatives via a cascade reaction, which distinguishes melatonin from classic antioxidants. This cascade enables one melatonin molecule to scavenge up to ten ROS versus classic antioxidants that are only capable of scavenging one or less ROS (Tan *et al*., 2003). Melatonin also has the capacity to be induced under moderate oxidative stress, further distinguishing it from classic antioxidants (Tan *et al*., 2015). The ability of melatonin to protect cells against oxidative stress more efficiently than other antioxidants has been demonstrated under *in vivo* conditions

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(Tunez *et al*., 2007; Ortiz *et al*., 2013). It has been demonstrated that excessive oxidative stress can lead to a rapid drop in circulating melatonin and is considered a protective mechanism for organisms against highly damaging oxidants. Therefore, melatonin can be categorized as a first line defensive molecule (Tan *et al*., 1998). The oxidative stress status of an organism modifies melatonin metabolism, whereby melatonin and its metabolites successively scavenge ROS via its free radical scavenging cascade (Tan *et al*., 2007). Aditionally, the toxicology effects of chronic melatonin exposure have been examined in both physiological and pharmacological amounts in humans and animals and it has been shown that the molecule is indeed nontoxic (Seabra *et al*., 2000).

Melatonin has also been suggested to be atheroprotective and may slow the progression of atherosclerotic development (Favero *et al*., 2014). Melatonin has been shown to act as a vasoconstrictor in the caudal artery and a vasorelaxant in the mesenteric artery and aorta (Slominski *et al*., 2012). The combination of vasoconstriction and vasodilation responses can be attributed to melatonin receptors mediating the response, since both MT1 and MT2 are present in the cardiovascular system and MT1 receptors mediate vasoconstriction and MT2 receptors mediate vascular dilatation (Masana *et al*., 2002). It has been shown that melatonin enhances the clearance mechanism of endogenous cholesterol into bile, while also inhibiting cholesterol synthesis and LDL accumulation, thus exerting a hypocholesterolaemic action (Hussein *et al*., 2007). Additionally, long term melatonin administration in a cholesterol fed rat model modified the fatty acid composition in plasma (Pita *et al*., 2002). A six week melatonin treatment also reduced plasma LDL cholesterol levels in Zucker diabetic fatty rats, and also reduced blood glucose and plasma lipids in streptozotocin-induced diabetic rats (Montilla *et al*., 1998). A known anti-inflammatory agent, melatonin could reduce molecular and cellular damage in blood vessels, even though the exact mechanisms have not yet been determined (Mauriz *et al*., 2013).

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1.7. Problem Identification

CVD is responsible for a rapidly growing number of deaths globally, and in South Africa where it is on the rise, specifically in the working age group (Steyn, 2007). Worldwide diseases associated with tobacco addiction kill more than six million people a year and it is expected that this number could increase to more than eight million people a year by 2030 (World Health Organization, 2011). Smoking, along with other cardiovascular risk factors, such as hypertension and diabetes, is associated with the development of ED, an early precursor of atherosclerosis (Cipollone *et al*., 2007).

ED can result in atherosclerosis, which, in turn, is the underlying pathology for many cardiovascular diseases, often resulting in myocardial infarction or stroke (Lusis, 2000). It has been shown that NO plays an important role in protection against the onset and progression of cardiovascular disease. The ability of the endothelium to synthesise and release NO is essential in regulating haemostasis, vessel tone, blood pressure and vascular remodelling (Naseem, 2005). Furthermore, the production of reactive oxygen species (ROS) and the resultant oxidative stress, are important mediators of the pathologic manifestations of endothelial dysfunction. ROS reduce and eliminate the protective abilities of NO, which in turn will lead to endothelial dysfunction (Barua *et al*., 2003). Experimental and clinical data indicate that exposure to nicotine increases oxidative stress and has the potential to induce endothelial dysfunction (Ambrose and Barua, 2004).

It has been shown that early endothelial changes, such as ED, are reversible (Hsueh *et al*., 2004). Therefore, it is clinically relevant to identify possible treatment modalities, such as antioxidant therapy, to counter the harmful effect of increased ROS production in order to restore the release of endothelioprotective NO. Protecting the endothelium will result in reduced or delayed atherogenesis, lowering the risk of cardiovascular mortality. Rooibos and melatonin are known to possess powerful antioxidant and cardioprotective effects and could help to reduce the harmful effects induced by nicotine. In addition, no adverse effects of rooibos or melatonin administration have been identified (McKay and Blumberg, 2007 and Pita *et al*., 2002).

Both rooibos and melatonin have been shown to display beneficial properties and could thus prove valuable in countering the harmful effect of nicotine as a risk factor for the development of cardiovascular disease. The current study aims to address the lack of studies investigating the putative protective effects of rooibos and melatonin in the context of nicotine induced cardiovascular injury.

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1.8. Research Aims and Objectives

In order to answer the above research questions, the following research approach was followed:

1.8.1. *In vivo* and *ex vivo* Pilot Study: Dose-response effect of nicotine exposure

Prior to commencement of the principal study an *in vivo* pilot study was undertaken to determine the dose-response effect of nicotine exposure for 6 weeks in adult male Wistar rats on the following parameters:

(i) Vascular function by means of contraction and relaxation studies in aortic rings harvested from treated and untreated male Wistar rats

(ii) Oxidative stress parameters by means of the thiobarbituric acid reactive substances (TBARS) assay in serum samples

(iii) Blood pressure measurements by means of the non-invasive tail-cuff method

1.8.2. Main *in vivo* and *ex vivo* study

Aim 1: To establish an *in vivo* model in which the effects of nicotine were assessed.

The optimal injury inducing-concentration of nicotine, as established in 1.8.1. was used in the main study. Nicotine-induced injury was assessed by investigating the following endpoints:

(a) Blood pressure measurements by means of the non-invasive tail-cuff method

(b) Vascular function in aortic tissue harvested from treated and untreated male Wistar rats. This is supported by an *in vitro* model of adult rat aortic endothelial cells (AECs), in which endothelial nitric oxide production and cell viability were assessed.

(c) Antioxidant enzyme activity and oxidative stress status. Superoxide dismutase (heart and liver tissue), catalase (heart and liver tissue) and glutathione peroxidase (heart tissue) assays were conducted to determine antioxidant enzyme activity and concentrations of conjugated dienes and TBARS (serum samples) analysed as measures of the oxidative stress status.

(d) Inflammatory markers, including TNF-α, IL-6 and CRP was analysed in serum samples

(e) Serum was analysed for total cholesterol, triglycerides and phospholipid concentrations in order to establish a serum lipid profile

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Aim 2: To determine whether co-treatment of nicotine with rooibos (unfermented and fermented) affects the nicotine-induced endpoints as described in Aim 1.

Aim 3: To determine whether co-treatment of nicotine with melatonin affects the nicotine-induced endpoints as described in Aim 1.

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CHAPTER 2: MATERIALS AND METHODS

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The study used healthy adult male Wistar strain rats throughout the pilot study and main study (Fig 2.1). The *in vivo* studies comprised a six week treatment period of subcutaneously administered nicotine or subcutaneously administered nicotine and rooibos (fermented or unfermented) or melatonin co-treatments. Rooibos and melatonin were given in the drinking water and appropriate subcutaneous and oral (fluid) controls were included. The *ex vivo* studies utilised aortas obtained from male Wistar rats after the abovementioned six week treatment period. The aortas were used in organ bath based vascular contraction-relaxation investigations. *Ex vivo* studies were supported by *in vitro* investigations in a model of adult rat aortic endothelial cells. The results of the investigations will follow in Chapter 3.

Figure 2.1. Healthy adult Wistar rat (www.scientificchicago.com).

2.1. General Materials Used

- (-) - Nicotine – Sigma-Aldrich (St. Louis, MO, USA) (CAS number: 54-11-5)
- Rooibos (Fermented and Unfermented) – Gift from the PROMEC unit of the South African Medical Research Council
- Melatonin – Sigma-Aldrich (St. Louis, MO, USA) (CAS number: 73-31-4)

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2.2. Animal Care

Ethics approval was received from Stellenbosch University; Project number SU-ACUM12-00041 and experiments were conducted according to 'The Revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008)'. Healthy, pathogen free adult male Wistar rats, weighing between 220-310 g at the start of the study, were housed at room temperature (23°C ± 2°C) in the Central Animal Housing Unit of the Faculty of Medicine and Health Sciences, Stellenbosch University. Animals were housed under normal 12 hour light and 12 hour dark cycles and had free access to standard rat chow and fluids. Animals were randomly assigned to treatment groups and individually caged in order to monitor fluid intake and were weighed on a regular basis. Animals receiving injectable treatments (nicotine and saline) were weighed daily and animals receiving only fluid as treatment (rooibos and melatonin) were weighed twice a week. Animals were euthanized with an overdose of sodium pentobarbital (160 mg/kg) by means of intra-peritoneal injection, before collecting blood, harvesting organs and excising aortas. Even though only heart and liver tissue were used in the study, the lungs, kidneys, pancreas and testes were also collected and frozen for possible use in future studies, thereby adhering to the principles of reduction and refinement of ethical animal use. The weight of each organ (heart, liver, lungs, kidneys, pancreas and testes) was also recorded.

2.3. Drug Administration

2.3.1. Physiological Saline

0.9 % Physiological saline was made up by dissolving 0.9 g NaCl in 100 ml double distilled water and thereafter autoclaved. The autoclaved saline solution was divided into 10 ml aliquots in sterile tubes and stored at 4°C. Aliquots were left on the bench to equilibrate to room temperature before it was administered to the rats via subcutaneous injection. Rats were weighed daily to determine the injection volume required. A new, sterile 26G needle was used for each injection.

2.3.2. Nicotine (1 mg/kg bw/day; 2 mg/kg bw/day; 5 mg/kg bw/day)

Nicotine was dissolved in sterile 0.9 % physiological saline (prepared as described under section 3.1.) according to the concentration required. Tubes of nicotine were wrapped in foil and stored at 4°C. Aliquots were left on the bench to equilibrate to room temperature before it was administered to the rats via subcutaneous injection. Rats were weighed daily to

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determine the injection volume required. A new, sterile 26G needle was used for each injection. Fresh nicotine was made up on a weekly basis.

2.3.3. 2% Rooibos (Fermented and Unfermented)

Fermented and unfermented rooibos were both made up from dried leaves according to the same procedure. Twenty g dried leaves were added to 1 l of boiling tap water and allowed to steep for 30 minutes, while stirring occasionally. The steeped solution was filtered through cheese cloth and then through number 4 filter paper (65 g/m^2). Lastly, the solution was filtered through a number 1 filter paper (84 g/m^2). Filtered rooibos was transferred to dark plastic bottles and stored at 4°C. Fresh rooibos was made up on a weekly basis. These concentrations are customarily used for tea making purposes and the protocol used conforms to the established method of rooibos preparation for experimental purposes (Marnewick *et al*., 2003). The rooibos solution served as the drinking fluid in the cages housing the rats assigned to the rooibos experimental groups.

2.3.4. Melatonin 4 mg/kg bw/day

Melatonin was weighed, dissolved in 1 ml absolute ethanol and then added to the drinking water at a final concentration of 0.05% (v/v) ethanol with melatonin. The final melatonin solution was then transferred to dark bottles. Fresh melatonin was made up on a daily basis. Rat fluid intake was recorded daily to ensure that the correct concentration of melatonin was received. The melatonin solution served as the drinking fluid in the cages housing the rats assigned to the melatonin experimental groups.

2.4. Soluble Solids, Total Polyphenols and Flavonoid Content Determinations of Rooibos

All determinations were performed for both 2% fermented rooibos and 2% unfermented rooibos, the concentration administered to rats throughout the study.

2.4.1. Soluble Solids

Soluble solid content of rooibos was determined gravimetrically. Clean, dry glass bottles were weighed prior to adding 1 ml aliquots of rooibos. The aliquots were dried at 70°C for 24 hours and subsequently placed in a desiccator for 24 hours. Glass bottles were again weighed after

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the drying period and initial weight of empty bottle subtracted to determine the soluble solid content. Determination of soluble solids were repeated six times on the same preparation.

2.4.2. Total Polyphenols

Total polyphenol content was determined by ARC Infruitec-Nietvoorbij, Post-Harvest Wine Technology Division, Stellenbosch, South Africa, using the Folin-Ciocalteu's phenol reagent. Briefly, 100 µl Folin-Ciocalteau reagent and 80 µl 7.5% (m/v) Na_2CO_3 were added to 20 µl blank (deiH_2O), standards (gallic acid; 10-100 mg/l in deiH_2O) or appropriately diluted rooibos in a 96 well plate and allowed to stand at room temperature for two hours (Arthur *et al*., 2011). Absorbance was read at 765 nm and expressed as mg gallic equivalents per mg soluble solids.

2.4.3. Flavonoid Content

2.4.3.1. Flavonol and Flavanol Determination

Flavonol content was spectrophotometrically determined at 360 nm, using a Spectronic® 20 Genesys^{TM} photospectrometer (Spectronic Instruments, Leeds, UK) and utilised quercetin as standard. Both quercetin and rooibos were diluted in 95% ethanol. Flavanol content was spectrophotometrically determined at 640 nm, using a Spectronic® 20 GenesysTM photospectrometer (Spectronic Instruments, Leeds, UK) using the 4-(dimethylamino)-cinnamaldehyde (DAC) reaction. DAC and rooibos were dissolved in HCl-MeOH (1:3). Catechin was dissolved in methanol to make a 0.05% solution and this served as standard for the flavanol determinations. For both flavonol and flavanol determinations the optimal dilution factor of rooibos was determined and subsequent analysis performed in triplicate (Ajuwon *et al*., 2013).

2.4.3.2. Analysis for Known Flavonoid Compounds

Known flavonoid compounds were determined by HPLC analysis by ARC Infruitec-Nietvoorbij, Post-Harvest Wine Technology Division, Stellenbosch, South Africa according to the HPLC method described in Joubert *et al*., 2012. Analyses were performed on an Agilent 1200 system (Agilent, Santa Clara, California, USA). Stock solutions of phenolic standards were prepared in dimethylsulfoxide (DMSO) at concentrations of 1 mg/ml and diluted with water according to experimental requirements and filtered through 0.22 µm polyvinylidene difluoride

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filters prior to use. Nine-point calibration curves were set up for all standards to test the linearity of the ultraviolet (UV)-diode array detector (DAD) response. The dihydrochalcones and phenylpyruvic acid-2-O-glucoside (PPAG) were quantified at 288 nm, while the flavones, flavonols, and ferulic acid were quantified at 350 nm. The calibration mixtures were injected at varying injection volumes, leading to levels of 0.025−1.20 µg on-column. Concentration ranges were selected to cover the different quantities of the compounds present in aqueous rooibos infusions. Linear regression, using the least-squares method (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA), was performed on the calibration curve data for each compound to determine the slope, y intercept, and correlation coefficients (r^2). Results are expressed as mg per litre.

2.5. *In vivo* and *Ex vivo* Investigations: Pilot Study: Dose-response Effect of Nicotine Exposure: 1 mg/kg bw/day Nicotine vs 2 mg/kg bw/day Nicotine

Prior to commencement of the main study, a pilot study was undertaken to assess the injury inducing properties of two previously described nicotine concentrations over a six week treatment period. Nicotine was subcutaneously administered at concentrations of 1 mg/kg bw/day or 2 mg/kg bw/day (Benowitz and Jacob, 1999, Balakumar *et al*., 2008b). Physiological saline, subcutaneously administered, served as vehicle control.

2.5.1. Treatment Groups

A total of 15 rats were divided into three groups (nicotine 1 mg/kg bw/day: n = 5; nicotine 2 mg/ml bw/day: n = 5; physiological saline: n = 5) (Fig 2.2).

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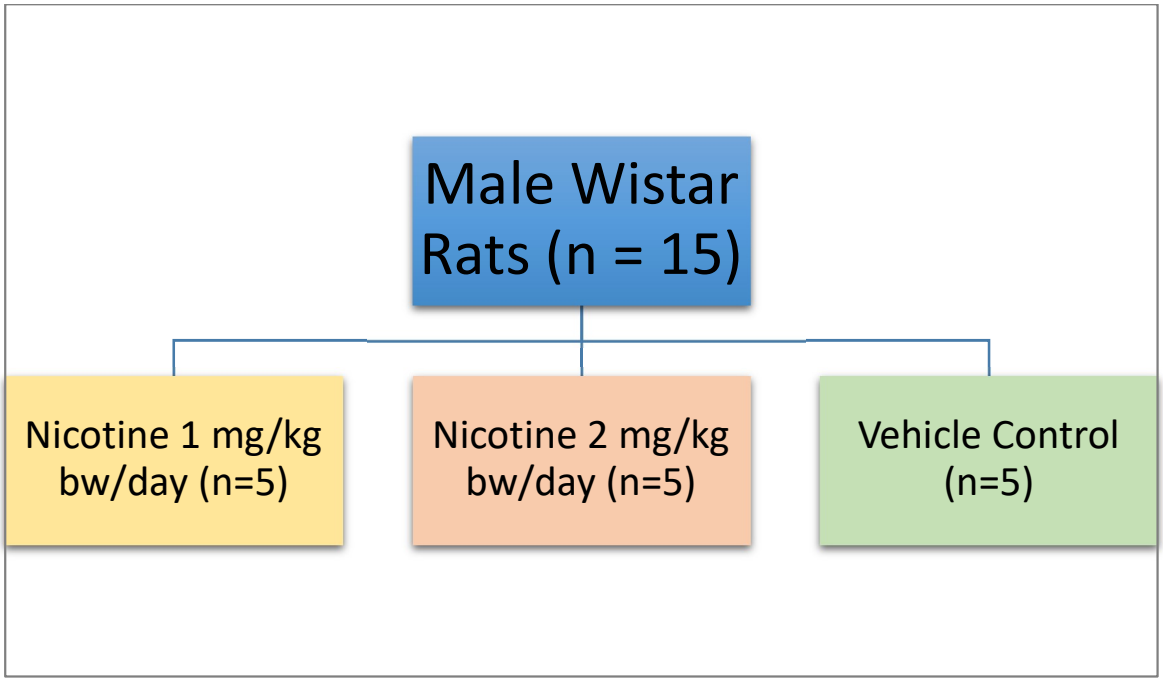

Figure 2.2. Treatment groups for the dose-response effect of nicotine – Pilot study. Nicotine and physiological saline (vehicle control) were administered subcutaneously.

2.5.2. Specific Endpoints Measured

- Blood pressure measurements by means of the non-invasive tail-cuff method
- Vascular function by means of aortic ring isometric tension studies
- Oxidative stress parameters by means of the TBARS assay kit (Cayman Chemical Company, Ann Arbor, MI, USA)

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2.6. *In vivo* and *Ex vivo* Investigations: Main Study

2.6.1. Treatment Groups

A total of 90 rats were divided into nine groups. Since neither 1 mg/kg bw/day nor 2 mg/kg bw/day nicotine concentrations resulted in sufficient injury, it was decided to opt for a higher concentration of nicotine, namely 5 mg/kg bw/day (Zimmerman and McGeachie, 1985). Nicotine and a saline vehicle control were administered subcutaneously. Rats were weighed on a daily basis to determine injection volume. Co-treatments consisted of 5 mg/kg bw/day administered with either 2% rooibos (fermented or unfermented) or 4 mg/kg bw/day melatonin. Control groups included a drinking control (tap water), as well as rooibos (fermented and unfermented) and melatonin groups. Rooibos (fermented and unfermented) and melatonin were supplied as drinking fluids. Each group contained 10 rats, resulting in a total of nine treatment groups (Fig 2.3). A schematic overview of the main in vivo study is given in figure 2.4.

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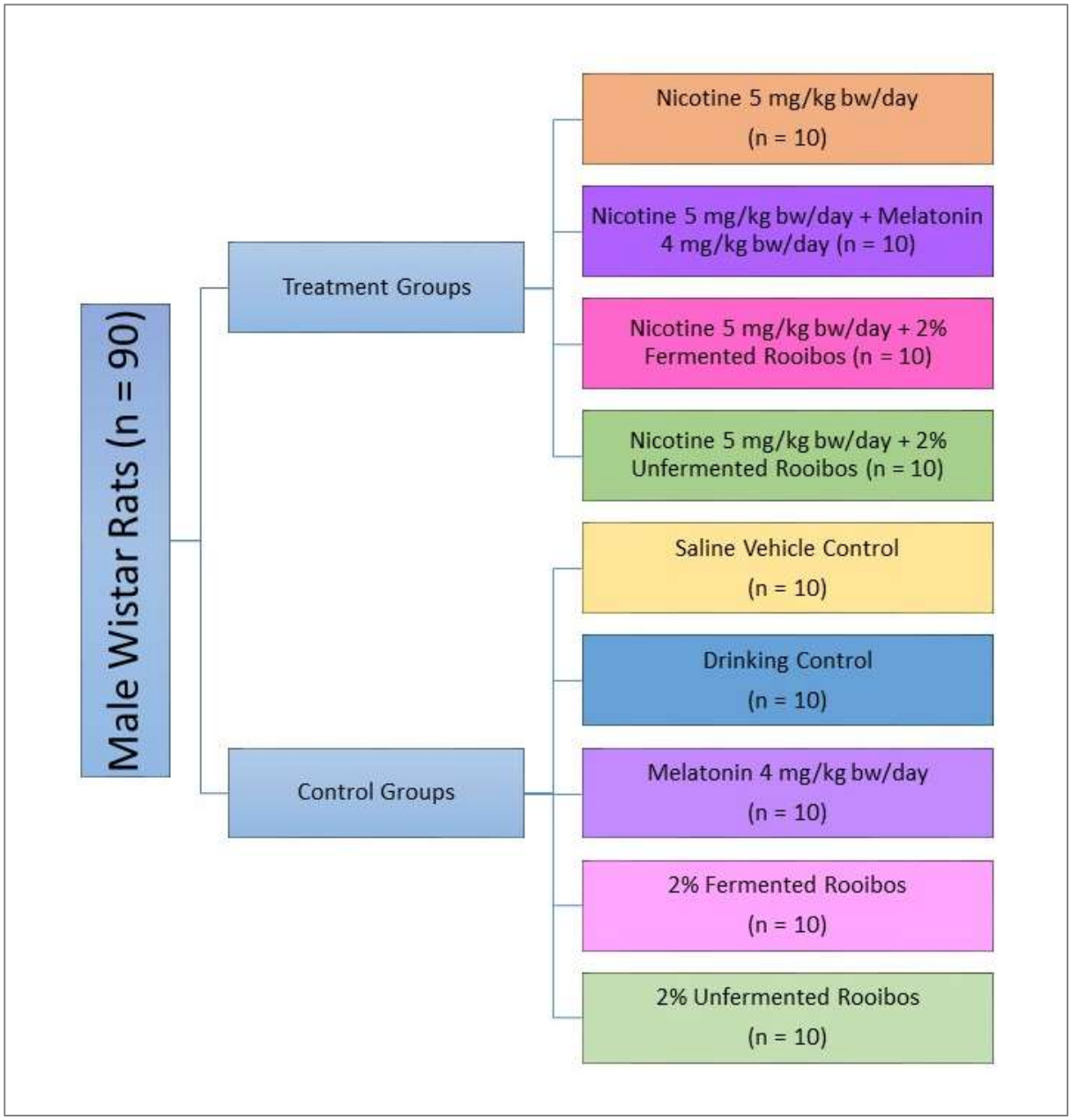

Figure 2.3. Treatment groups for the main *in vivo* study.

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2.6.2. Specific Endpoints

- Blood pressure measurements by means of the non-invasive tail-cuff method
- Vascular function by means of aortic ring isometric tension studies, complemented by *in vitro* studies in cultured adult rat aortic endothelial cells
- Antioxidant enzyme activity and oxidative stress status determinations
- Inflammatory marker determinations
- Serum lipid measurements

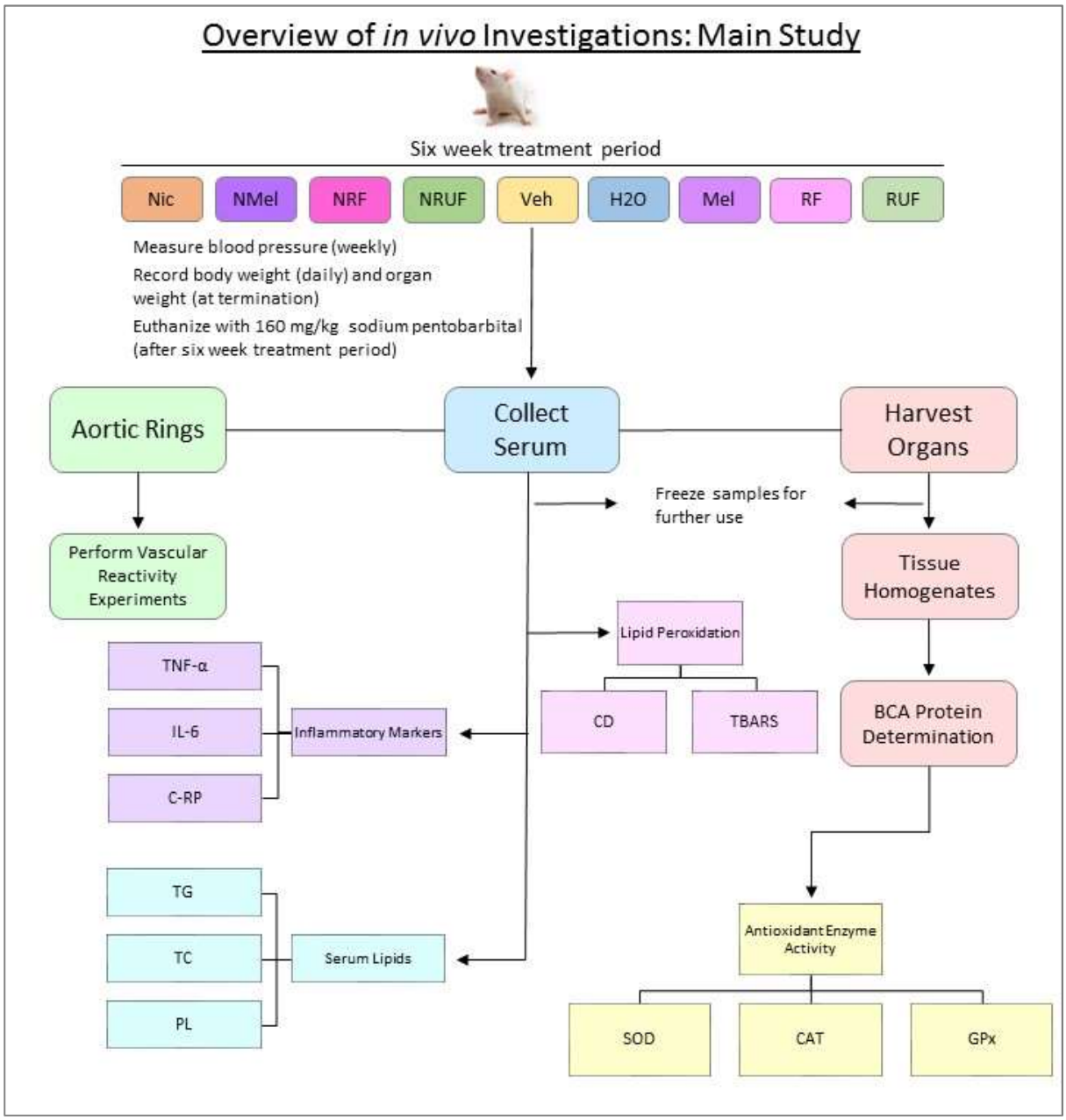

Figure 2.4. Schematic overview of the *in vivo* investigations carried out in the main study.

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2.7. General Experimental Procedures

2.7.1. Blood Pressure Measurements

Blood pressure of rats was measured non-invasively on a weekly basis by means of the volume-pressure recording (VPR) tail-cuff method, which calculates blood pressure by determining tail blood volume with a sensor and occlusion tail cuff (CODA^{TM} System, Kent Scientific) (Fig 2.5). The rats were acclimatized to the apparatus for one week prior to measurements.

Figure 2.5. Blood pressure measurement in a male Wistar rat using the CODA^{TM} system (Kent Scientific). Rat in rodent holder on heating platform. VPR and occlusion cuffs are attached to the tail.

2.7.2. Sample Collection

Rats were fasted overnight and euthanized with an overdose of sodium pentobarbital (160 mg/kg). Blood was collected and allowed to clot on ice for 30 minutes. After 30 minutes the blood was centrifuged at 1200 g for 10 minutes at 4°C and serum collected. The serum was aliquoted and stored at -80°C for further analysis. Organs, including the heart, lungs, liver, pancreas and testes were harvested, rinsed, weighed and flash frozen in liquid nitrogen. Tissue samples were stored at -80°C for further analysis. The aorta was excised, cleaned of excess tissue and perivascular fat, and used immediately for vascular contraction / relaxation experiments. The sample collection procedure is described in figure 2.6.

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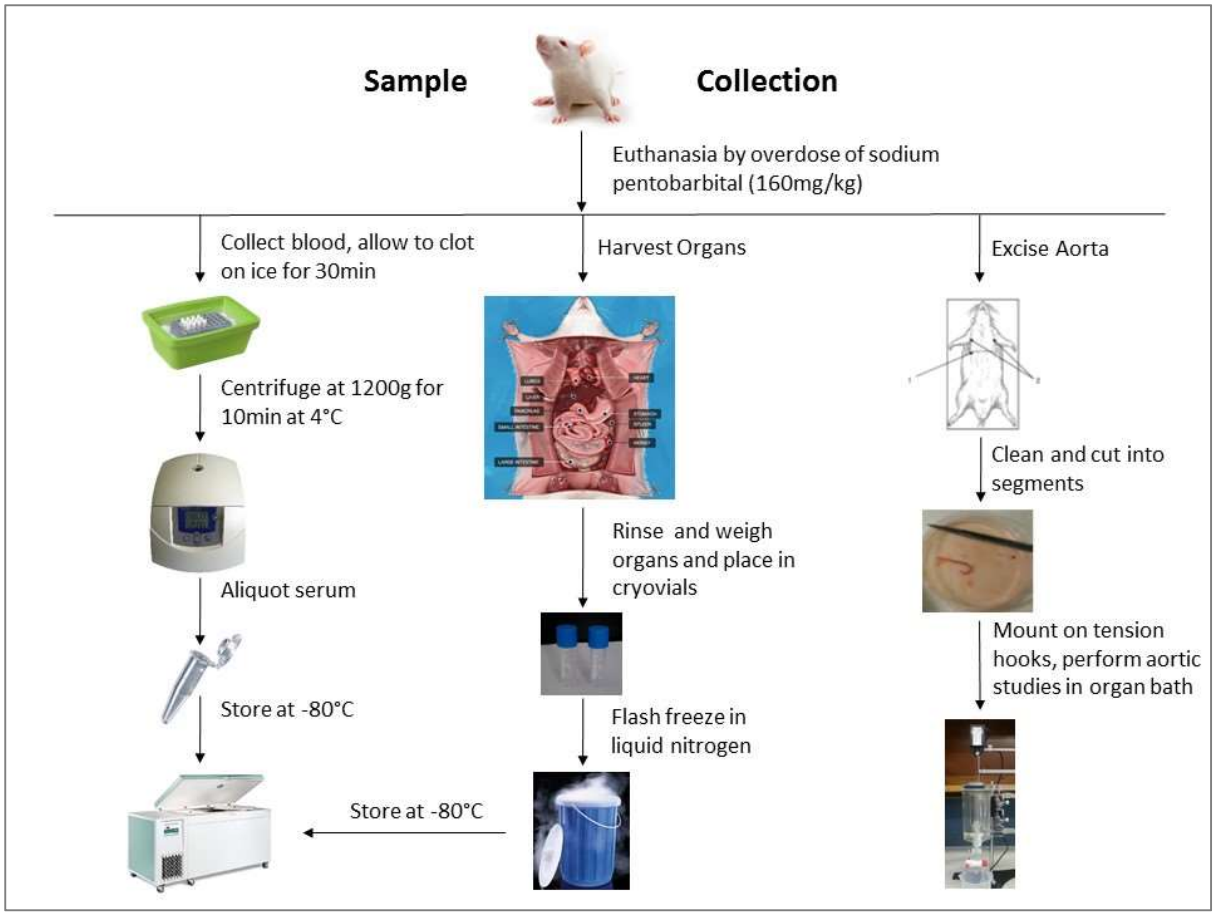

Figure 2.6. Sample collection procedure.

2.7.3. *Ex vivo* Investigations: Aortic Ring Isometric Tension Studies

2.7.3.1. Excision and Mounting of Rings

The thoracic aorta was excised and immediately placed in ice cold Krebs Henseleit buffer (KHB, composition in mM: NaCl 119, $NaHCO_3$ 25, KCl 4.75, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 0.6, Na_2SO_4 0.6, $CaCl_2.H_2O$ 1.25 and glucose 10). All connective tissue and perivascular fat were removed and the aorta was cut into 3-4 mm segments. The cleaned aortic ring was mounted onto two stainless steel hooks and lowered into a 25 ml organ bath (AD Instruments, Bella Vista, New South Wales, Australia) (Fig 2.7 and Fig 2.8). The organ bath contained oxygenated (95% O_2 and 5% CO_2) KHB. The tension of the aortic ring was recorded (grams of tension) with an isometric force transducer (TRI202PAD, Panlab, ICornellà, BCN, Spain) and the data analysed with LabChart 7 software (Dunedin, New Zealand).

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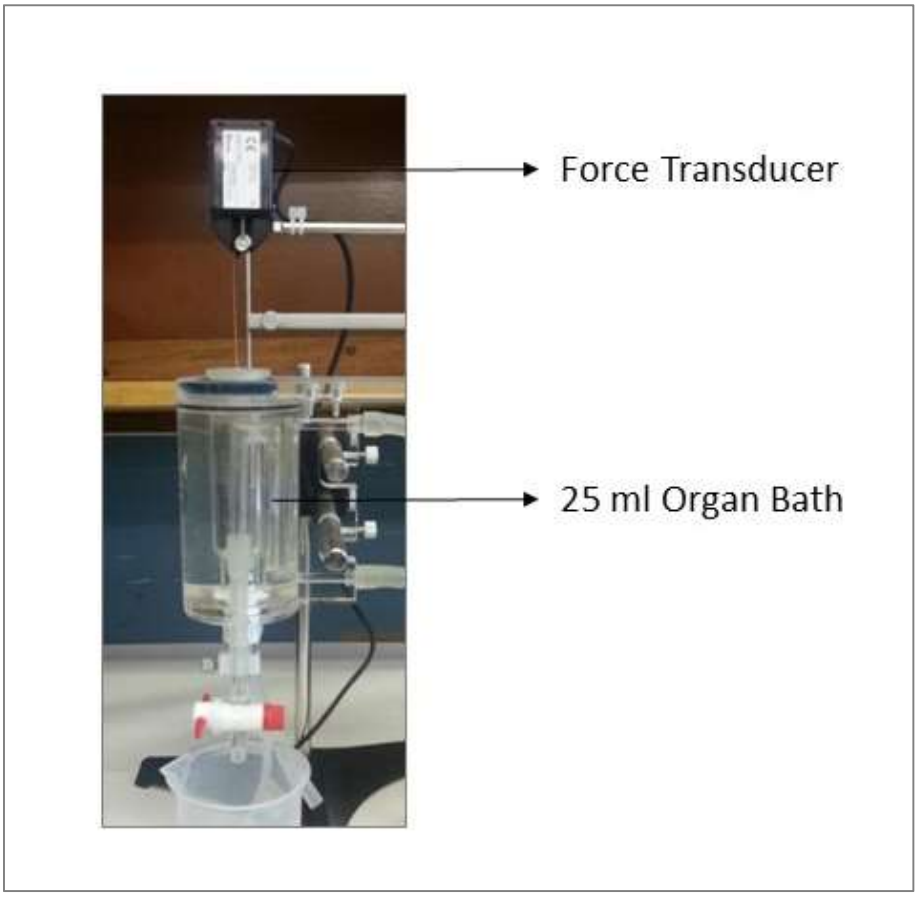

Figure 2.7. Organ bath and transducer used for aortic ring studies.

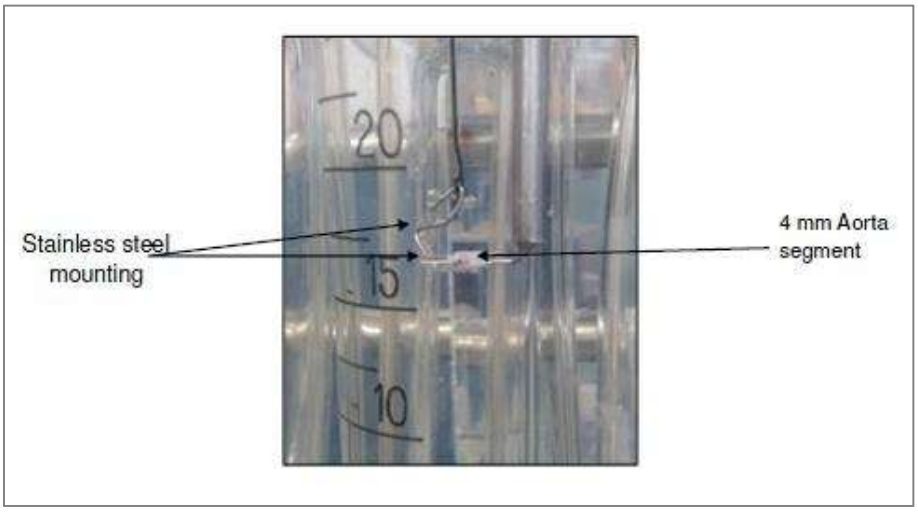

Figure 2.8. Aortic ring suspended between two stainless steel hooks and lowered into organ bath (from Loubser D, MSc thesis, Stellenbosch University, April 2014).

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2.7.3.2. Experimental Protocol

The experimental protocol was based on a modified protocol of isometric tension measurement (Privett *et al*., 2004). The protocol can be divided into an initial stabilisation phase, followed by a first round of contraction and relaxation in order to establish the functionality of the endothelium. The first round of contraction and relaxation was followed by a second stabilisation phase. This was followed by a round of cumulative contraction and cumulative relaxation.

2.7.3.2.1. First Round Stabilisation

The mounted aortic ring was lowered into the organ bath and thereafter it was subjected to a 30 minutes stabilisation period. The resting tension of the aortic ring was increased to 1.5 g during this period. The KHB was changed every 10 minutes with pre-warmed KHB (37°C).

2.7.3.2.2. First Round Contraction / Relaxation

A first round of contraction with phenylephrine (Sigma-Aldrich, St. Louis, MO, USA) (100 nM) and relaxation with acetylcholine (Sigma-Aldrich, St. Louis, MO, USA) (10 µM), added directly into the organ bath, was carried out in order to test the functionality of the endothelium. Phenylephrine (Phe) is an α-adrenergic receptor agonist which leads to smooth muscle contraction though its direct action on the vascular smooth muscle cells, whereas acetylcholine (ACh) binds to endothelial surface receptors, which results in increased intracellular calcium levels and consequently eNOS activation. The subsequently released NO diffuses into the underlying smooth muscle cells leading to relaxation. ACh was added to the organ bath after the Phe-induced contraction reached a plateau. Rings that showed at least a 70% relaxation of maximum Phe-induced contraction were deemed viable and used for further investigations.

2.7.3.2.3. Second Round Stabilisation

Following the first round of contraction/relaxation the organ bath was rinsed three times with KHB in order to flush out Phe and ACh. The rings were again stabilised for 30 minutes at 1.5 g, as described in 2.7.3.2.1.

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2.7.3.2.4. Second Round Cumulative Contraction / Relaxation

Phe was used to induce cumulative aortic ring contractions. The step-wise increase in total Phe concentration was as follows: 100 nM, 300 nM, 500 nM, 800 nM, 1 µM. After the maximum Phe-induced contraction was reached, ACh was cumulatively added to induce relaxation. The step-wise increase in total ACh concentration was as follows: 30 nM, 100 nM, 300 nM, 1 µM, 10 µM. The final concentration of ACh resulted in maximum % relaxation of contraction and was the endpoint of the experiment. Figure 2.9 shows a timeline of the experimental protocol.

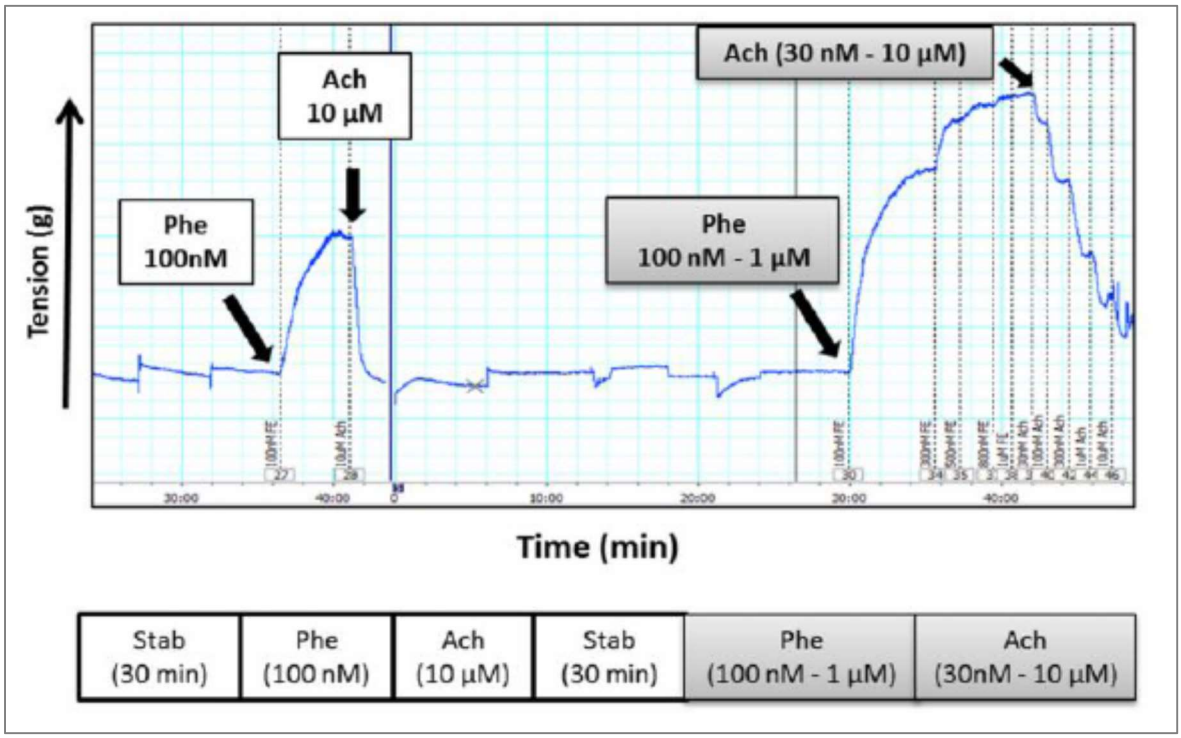

Figure 2.9. Timeline of the aortic ring studies experimental protocol. (From Vascular Reactivity: Aortic ring protocol, Laboratory Manual, Stellenbosch University, Westcott C, 2015.)

2.7.3.3. Statistical Analysis

Data were analysed using GraphPad Prism® 5 software (GraphPad Software, San Diego, CA, USA). All aortic ring isometric tension data are expressed as the % contraction from a resting tension of 1.5 g or % relaxation of maximum contraction respectively. Data were statistically analysed by means of two-way analysis of variance, followed by the Bonferroni post-test. Differences with a p-value < 0.05 were considered statistically significant.

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2.7.4. *In vitro* Investigations

2.7.4.1. General Materials Used

- Endothelial cell growth medium (EGM-2) – Clonetics (Cambrex Bio Science, Walkersville, USA)
- Attachment factor (solution containing gelatine) – Life Technologies (Carlsbad, California, USA)
- Trypsin (solution containing 2.5% trypsin) - Life Technologies (Carlsbad, California, USA)
- Foetal bovine serum (FBS) – Highveld Biological (Lyndhurst, RSA)
- Nicotine – Sigma-Aldrich (St. Louis, MO, USA) (CAS number: 54-11-5)
- Rooibos (Fermented) – Gift from the PROMEC unit of the South African Medical Research Council

2.7.4.2. Aortic Endothelial Cells (AECs)

Adult rat AECs cultures were purchased commercially from VEC Technologies (Rensselaer, New York, USA). Cells were grown to confluency in a standard tissue culture incubator (Forma Series II, Thermo Electron Corporation, Waltham, MA, USA) at an atmospheric composition of 21% O_2, 5% CO_2, 40-60% humidity and temperature was maintained at 37°C.

2.7.4.3. Passaging Procedures

Cells were received in 75 cm^2 tissue culture flasks, supplied with fresh growth media (EGM-2) and allowed to grow until confluent in a standard tissue culture incubator under the conditions described above. Cells received fresh EGM-2 every second day thereafter. The growth medium was supplemented with 10% FBS and standard endothelial growth factors (vascular endothelial growth factor (VEGF), human epidermal growth factor (hEGF), long chain human insulin-like growth factor (R^3-IGF-1), human fibroblastic growth factor (hFGF), hydrocortisone, antibiotics (gentamicin and amphotericin B) and ascorbic acid), according to the manufacturer's instructions. Confluency was evaluated by microscopic observation, demonstrating that the cells were evenly distributed and completely covered the growth area of the tissue culture flask. When confluency was reached cells were washed with phosphate buffered saline (PBS) and incubated with pre-warmed (37°C) solution containing 2.5% trypsin

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to allow cells to detach. The isolated cell-trypsin mixture was immediately transferred into 15 ml conical tubes containing fresh EGM-2 and centrifuged for three minutes at 1000 rpm. The supernatant was removed and the pellet resuspended in fresh EGM-2. Passaging to the next generation was performed in a 1:2 ratio. Cell aliquots were stored in a special "freezing" medium of 90% FBS, 5% cell growth medium and 5% DMSO, and stored in liquid nitrogen for subsequent use.

Cell aliquots taken from liquid nitrogen for experimental purposes were allowed to thaw in the tissue culture incubator (10-15 min) and seeded in two, attachment factor coated, 35 mm petri dishes. Fresh growth medium was given the next day and cells were allowed to grow until confluent. Cells were then passaged in the manner described above, by which passaging to the next generation was performed in a 1:2 ratio. According to this method, a P1 cell line could result in 128 petri dishes, a P2 cell line in 64 petri dishes and a P3 cell line in 32 petri dishes.

2.7.4.4. General *In vitro* Methods

2.7.4.4.1. Flow Cytometric Analysis

Flow cytometric analysis of all fluorescent probes utilised in the *in vitro* study were performed on a Becton-Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ, USA) and data was analysed by means of the WinMDI® 2.9 software package (Purdue University Cytometry Laboratories, West Lafayette, USA). A total of 10 000 cells were analysed for each experimental sample, while control and appropriate positive controls were added for each probe. Cell populations (healthy cells, excluding debris and non-cellular particles) of control samples were gated and gate coordinates used for experimental samples in the same flow cytometry session. The probes used in this study are listed in Table 2.1.

Table 2.1. Fluorescent probes used in the *in vitro* study.

Endpoint	**Flow cytometric analyses**
NO production	4,5-diaminofluorescin-2 diacetate (DAF-2/DA) fluorescence
Apoptosis	Annexin-V fluorescence
Necrosis	Propidium iodide (PI) fluorescence

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2.7.4.4.2. Measurement of Nitric Oxide Production

NO production was measured by 4,5-diaminofluorescin-2 diacetate (DAF-2/DA) fluorescence (Calbiochem, San Diego, CA, USA), which is a non-fluorescent cell permeable reagent capable of measuring free NO and NOS activity in living cells. After entering the cell, the diacetate groups on the DAF-2/DA are hydrolysed by cytosolic esterases, which release the DAF-2. NO converts the non-fluorescent dye to its fluorescent triazole derivative, DAF-2T. DAF-2T fluorescence can be analysed in flow channel 1 of the flow cytometer. The protocol for measurement of NO production by DAF-2/DA has previously been established in our laboratory (Strijdom *et al*., 2004 and 2006).

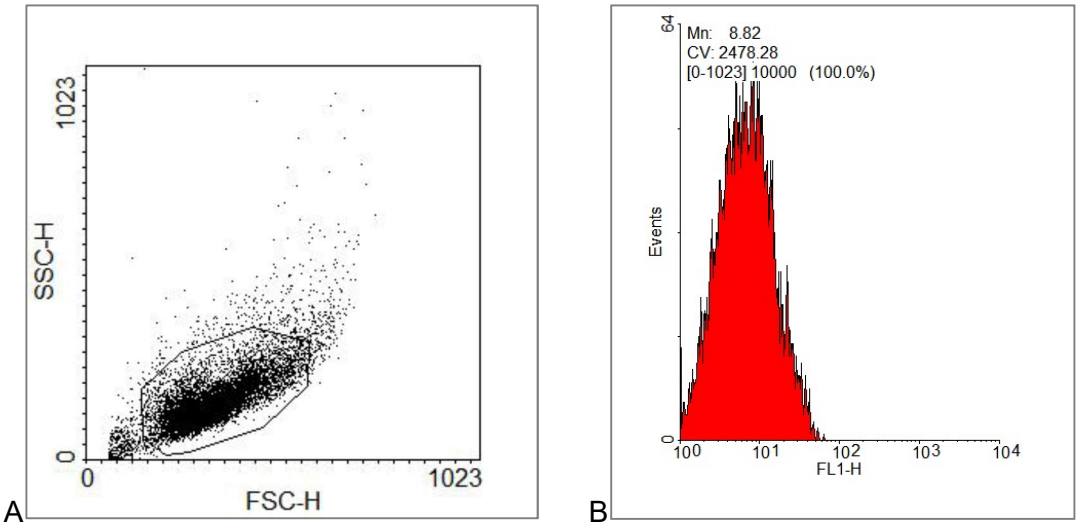

Figure 2.10. A shows a scatterplot with gating of the population of interest. Only the events within the gate will be represented on the histogram. B shows a histogram of FL1-H (x-axis), in this case a mean fluorescence intensity (Mn) of 8.82 was obtained.

2.7.4.4.3. Experimental Protocol and Data Analysis

When cells had reached confluency, petri dishes were randomly assigned to control, positive control and treatments groups. One petri dish was reserved to serve as absolute control and remained free of any treatments and the probe. After the treatment period, cells were washed twice with PBS and incubated with 10 µM DAF-2/DA probe for two hours. The positive control, 100 µM diethylamine NONOate diethylammonium salt (DEA/NO) (Sigma-Aldrich, St Louis, MO, USA), which is an NO donor, was added to positive control petri dishes for the last 30 min of the two hour DAF-2/DA incubation period (Fig 2.12). After the incubation period, the probe was aspirated, cells washed with PBS and trypsinized into a 15 ml conical tube

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containing cell staining buffer. Cell staining buffer (Bio-Legend, Biochom-Biotech, San Diego, CA, USA) was used at this stage of experimentation, since it is colourless. Cell growth media has a pink colour, which could interfere with the flow cytometric analysis, which is based on fluorescent colour emission. Cell suspensions were centrifuged for three minutes at 1000 rpm, after which the supernatant was aspirated. Cell pellets were resuspended in PBS and transferred to FACS tubes for flow cytometric analysis. A schematic representation of the protocol is given in figure 2.11.

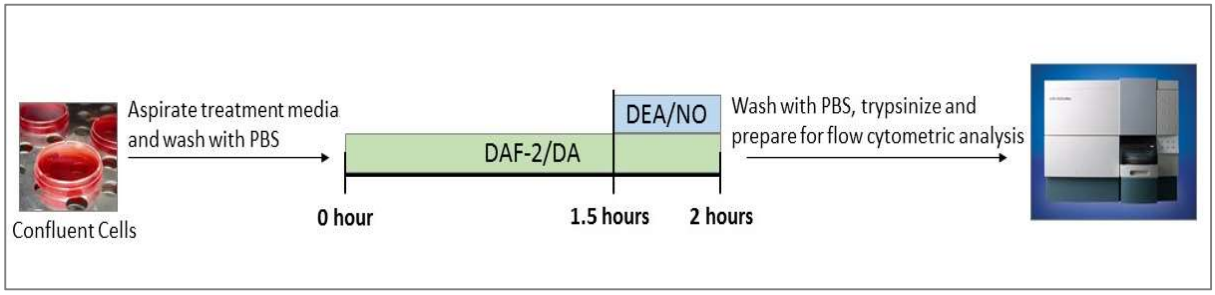

Figure 2.11. Schematic representation of the protocol used for DAF-2/DA probe and the positive control DEA/NO.

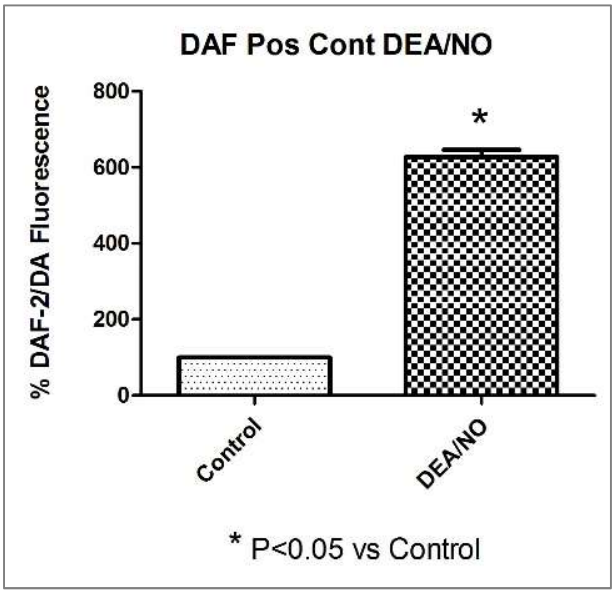

Figure 2.12. DEA/NO (100 µM, 30 min) significantly increased mean DAF-2/DA fluorescence when compared to controls. DEA/NO served as positive NO control in the study.

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2.7.4.4.4. Measurement of Apoptosis and Necrosis (Cell Viability)

Cell viability was assessed by Anexa Fluor® 647 Annexin V conjugate, which is a marker of apoptosis, and propidium iodide (PI) which is a marker of necrosis (Bio-Legend, Biochom-Biotech, San Diego, CA, USA). For experiments in which only necrosis was measured, PI was obtained from Sigma-Aldrich (St. Louis, MO, USA). Annexin V binds to phosphatidylserine on the outer membrane, enabling it to identify cells undergoing apoptosis. Annexin V fluorescence is measured in flow channel 4 of the flow cytometer. PI is an intercalating agent, which can enter the nucleus of a cell when membrane integrity is lost due to cell death (Wilkins *et al*., 2002). PI fluorescence is measured in flow channel 2 of the flow cytometer.

2.7.4.4.5. Experimental Protocol and Data Analysis

When cells had reached confluency, petri dishes were randomly assigned to control, positive control and treatments groups. A positive control for cell death, namely distilled H_2O was added for five min. After the treatment period, cells were washed twice with PBS and trypsinized into 15 ml conical tubes containing cell staining buffer and centrifuged for 2 min at 700 rpm. After centrifugation, the cell staining buffer was aspirated and the cell pellet resuspended in binding buffer (Bio-Legend, Biochom-Biotech, San Diego, CA, USA) and the cell suspension transferred to a FACS tube. Annexin V (final concentration: 5 µM) and PI (final concentration: 5 µM) were added to the tube and incubated for 15 min in the dark. Flow cytometric analysis was carried out after the incubation period. When using Annexin V and PI in the same experiment, parameters could be simultaneously measured and analysis carried out by creating quadrants of the gated populations. The use of quadrants allowed for discrimination between apoptotic and necrotic populations (Fig 2.13 and Fig 2.14). Distilled H_2O served as positive control for necrosis, whereby cells were washed with PBS and then treated with dH_2O for five minutes. H_2O is an appropriate control for necrosis, since it will diffuse across a semi-permeable membrane from an area with low osmolarity (outside the cell) to an area of high osmolarity (inside the cell). As H_2O moves into the cell, the cell will swell and the cell membrane rupture, allowing PI to enter and bind DNA (Fig 2.15).

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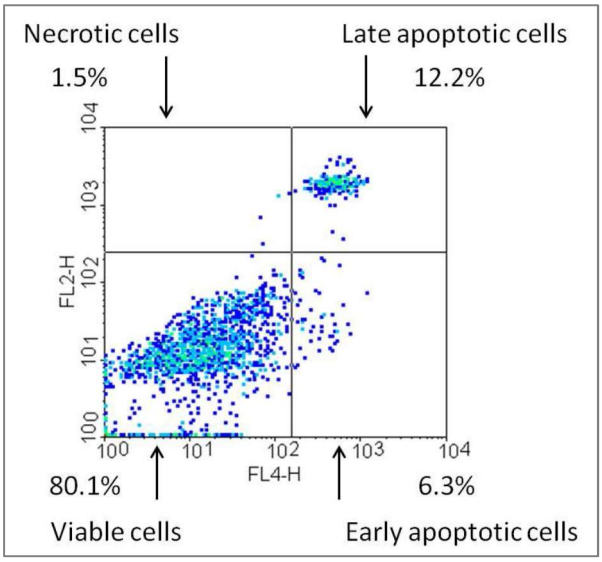

Figure 2.13. A density plot showing the quadrants used for simultaneous analysis of necrosis and apoptosis. PI was measured in the FL2-H channel (y-axis) and Annexin V in the FL4-H channel (x-axis).

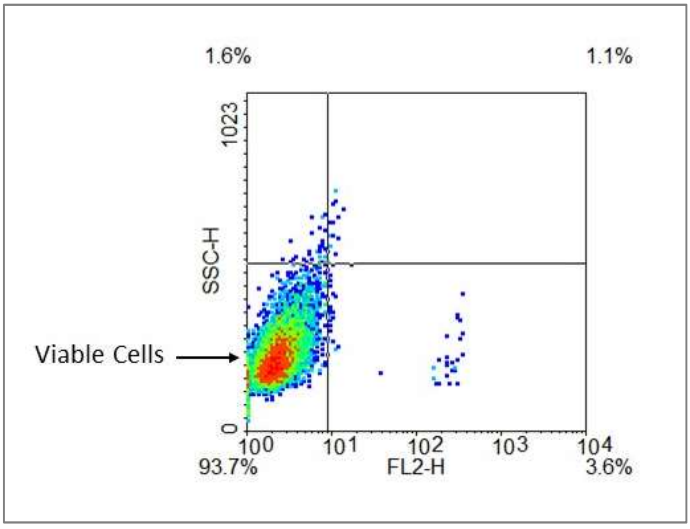

Figure 2.14. Density plot showing quadrants used for data analysis of propidium iodide-stained necrotic cells only. The lower left quadrant shows the percentage viable cells and the lower right quadrant shows the percentage necrotic cells.

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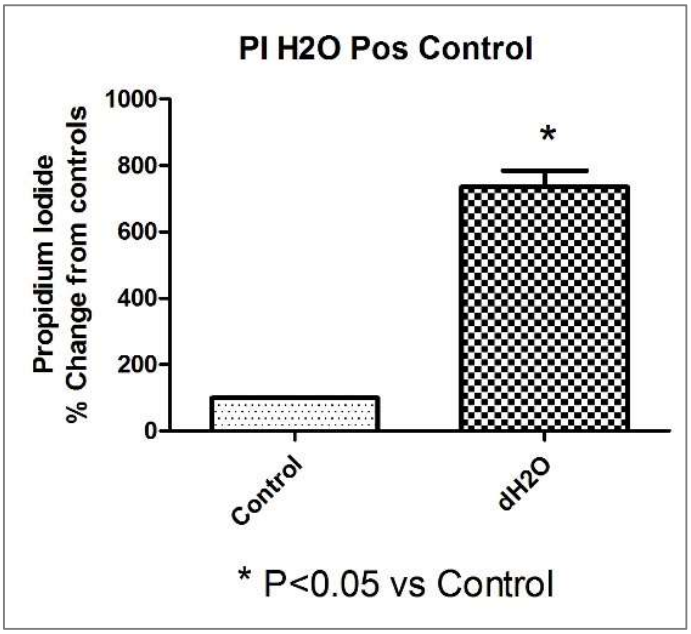

Figure 2.15. dH_2O served as positive control for PI experiments, resulting in a significant increase in PI fluorescence when compared to controls.

2.7.4.5. Nicotine and Rooibos Experimental Protocols

2.7.4.5.1. Nicotine Dose-response Experiments

An optimal injury inducing concentration of nicotine had to be established for AECs. Nicotine was diluted with PBS and the following concentrations were tested for resultant decline in NO production and increase in necrosis and apoptosis, when compared to controls: 1 µM; 10 µM and 100 µM. Nicotine at a concentration of 1 µM is typically found in the bloodstream of those who smoke one pack of cigarettes a day (Dasgupta *et al*., 2006; Alamanda *et al*., 2012). A treatment period of 24 hours was used for all experiments. NO production and necrosis and apoptosis were determined according to the protocols described in section 2.7.4.4.2 and 2.7.4.4.4.

2.7.4.5.2. Rooibos Dose-response Experiments

An optimal concentration of fermented rooibos (RF) for use on AECs had to be established. A treatment period of 24 hours was used for all experiments. NO production and necrosis were determined according to the protocols described in section 2.7.4.4.2 and 2.7.4.4.4. RF concentrations ranging between 0.015 mg/ml and 0.5 mg/ml were tested.

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2.7.4.5.3. Preparation of Fermented Rooibos (RF)

RF was prepared according to the protocol described in section 2.3.3. RF was freeze dried in a FreeZone6 (Labconco, Kansas City, MO, USA) freeze drier to remove the aqueous fraction. 100 ml RF was used per 250 ml vacuum flask and shelled in liquid nitrogen. RF was freeze dried overnight (± 24 hours), transferred to 15 ml conical tubes and stored in a desiccator. Freeze dried RF was made up to a 20 mg/ml stock solution in cell culture media and further diluted in cell culture media according to the concentration required.

2.7.4.6. Nicotine with Fermented Rooibos (RF) Pre-treatment: Administration and Experimental Layout

Cells were co-treated with nicotine and RF after determining the optimal concentrations of nicotine and RF for AECs, to determine if RF could reduce the damage caused by nicotine. NO production and necrosis were measured as described in section 2.7.4.4.2 and 2.7.4.4.4. Cells were pre-treated with RF for one hour, after which nicotine was added for a further 24 hours.

2.7.4.7. Statistical Analysis

All flow cytometric data were calculated as mean ± standard error of the mean. Controls were adjusted to 100 % and values expressed as a % of the control. The Student's t-test or one-way analysis of variance (with Bonferroni multiple comparison test) were used to determine statistical significance. Data were analysed using GraphPad Prism® 5 software (GraphPad Software, San Diego, CA, USA) and differences with a p-value of <0.05 regarded as statistically significant. Experiments were repeated at least once to result in a sample size of 6-8 per group. Sample sizes are indicated below each graph in Chapter 3.

2.7.5. Tissue Homogenate Preparation

Heart and liver homogenates were prepared from the snap-frozen tissue samples obtained after euthanasia and subsequently stored at -80°C (described in Section 7.2). Tissue homogenates were prepared in microcentrifuge tubes using the Bullet Blender 24 (Next Advance, NY, USA). The recommended Next Advance protocols for heart and liver homogenization for small samples were used as guidelines. Briefly, a small amount of tissue (± 100 mg) and equal amount of homogenization beads were weighed and two volumes of buffer (50 mM sodium phosphate, pH 7.5) added. For liver tissue 0.5 mm zirconium oxide

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beads were used and for heart tissue 1.6 mm stainless steel beads were used. Samples were kept cold at all times and homogenization took place at 4°C. Samples were homogenized for one minute and then rested for one minute to ensure samples remained cold. After satisfactory homogenization was achieved, samples rested on ice for 30 minutes. Samples were then centrifuged at 12000 rpm for 20 minutes, after which the supernatant was transferred to a clean microcentrifuge tube. Homogenates were diluted with buffer and aliquots stored at -80°C. An additional centrifugation step was employed for heart homogenates to allow for separation of cytosolic and mitochondrial fractions. The original supernatant was transferred to a clean microcentrifuge tube and centrifuged at 14000 rpm for 20 minutes. The supernatant (cytosolic fraction) was transferred to a clean microcentrifuge tube, diluted with buffer and aliquots stored at -80°C. The pellet (mitochondrial fraction) was resuspended in buffer and washed by means of centrifugation. The resulting pellet was diluted with buffer and aliquots stored at -80°C. An overview of the homogenization process is given in figure 2.16.

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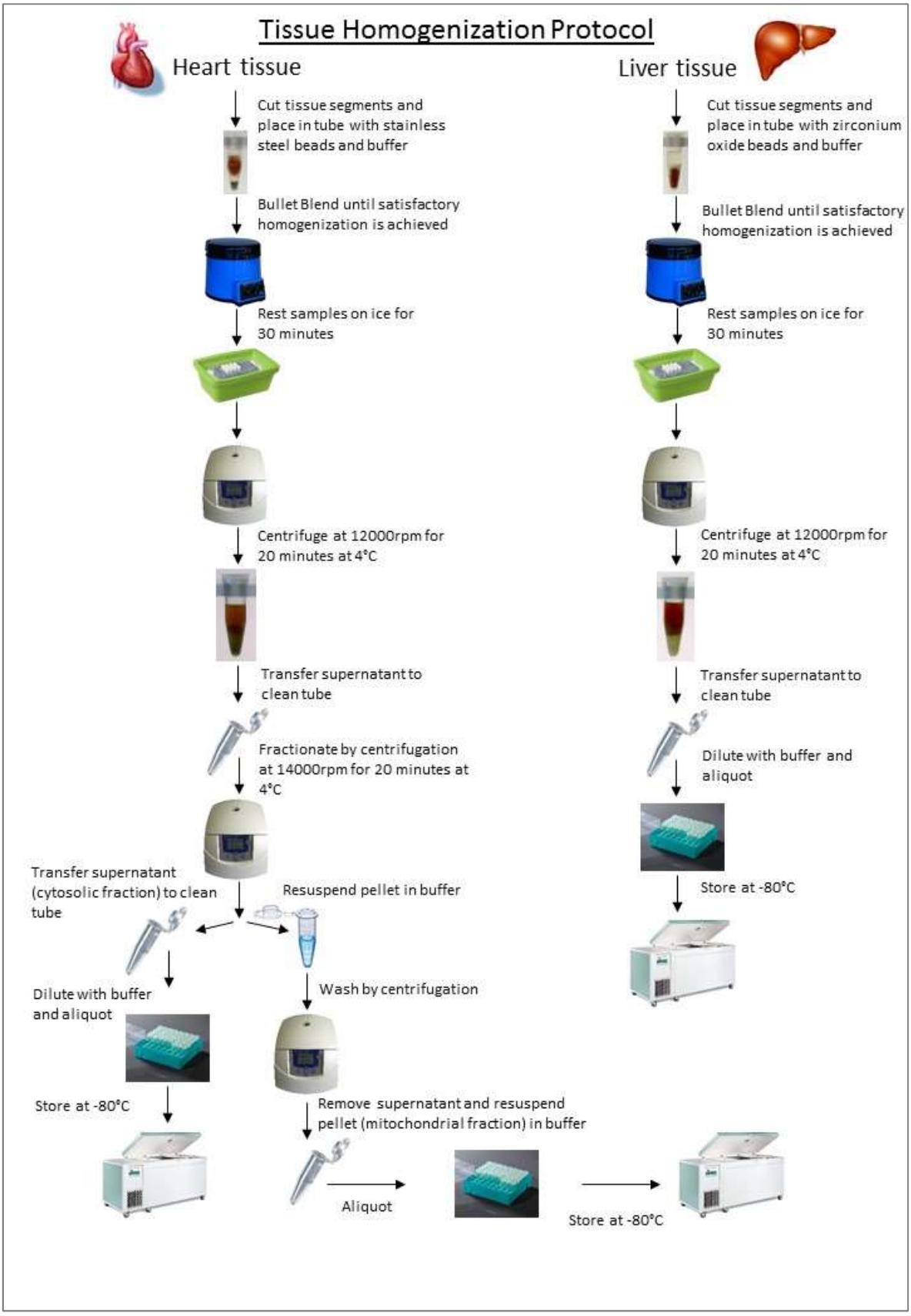

Figure 2.16. Heart and liver tissue homogenization using the Bullet Blender 24 by Next Advance (NY, USA).

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2.7.6. Protein Quantification of Homogenates

For the determination of the protein quantity of each tissue homogenate, the bicinchoninic acid (BCA) protein assay kit (BCA1 and B9643) by Sigma-Aldrich (St Louis, MO, USA) was utilised. The assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}, whereby the amount of reduction is proportional to the amount of protein present. BCA forms a stable purple complex with Cu^{1+} in alkaline environments, which forms the basis of the colourimetric assay. The micro-method, as described in the kit insert, makes use of a 96 well plate, was used for all assays. The protocol provided with the BCA assay kit was used. Reagent A (BCA solution) and Reagent B (copper (II) sulfate pentahydrate 4% solution) were mixed in a 50:1 ratio to form the green BCA Working Reagent. Bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) served as standard. A stock of 1 mg/ml was diluted with deionized water (deiH_2O) to concentrations ranging from 0.2 mg/ml to 0.8 mg/ml to derive a standard curve (Fig 2.17). Tissue homogenates were diluted with deiH_2O to bring them within the linear concentration range of 200-1000 µg/ml of protein. To each well, 25 µl of protein (standard or sample) and 200 µl BCA Working Reagent was added. A blank was also included. Plates were incubated at 37°C for 30 minutes and absorbance read at 562 nm in a FLUOstar Omega Microplate Reader (BMG Labtech, Offenburg, Germany). The assay was performed in triplicate for all samples.

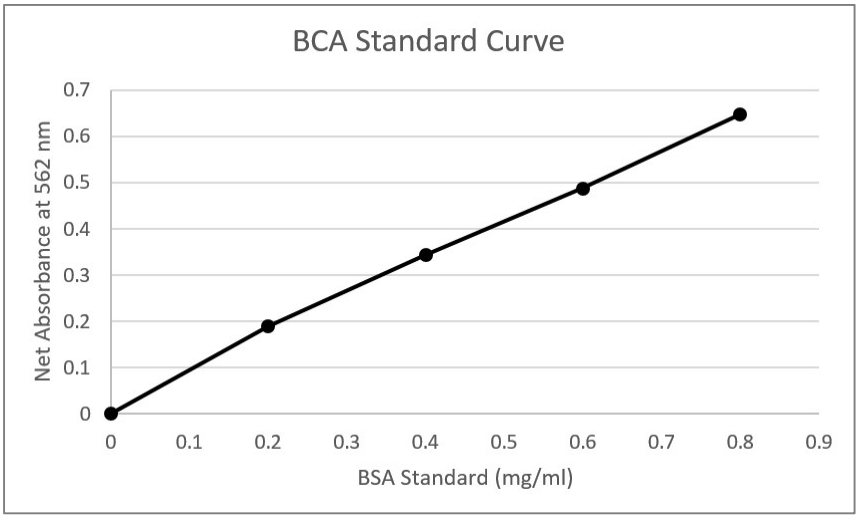

Figure 2.17. Example of BCA assay standard curve. BSA served as standard and absorbance was read at 562 nm.

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2.7.7. Antioxidant Enzyme Activity and Oxidative Stress Status

2.7.7.1. Superoxide Dismutase

Superoxide dismutases (SODs) form a crucial part of the cellular antioxidant defence mechanism. These metalloenzymes catalyse the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide through the following reaction: $2O_2^{\bullet-} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$. The Superoxide Dismutase Assay Kit (706002) by the Cayman Chemical Company (Ann Arbor, MI, USA) was utilised for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (Fig 2.18). The assay measured all three types of SOD (Cu/Zn, Mn, and FeSOD) and one unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide free radical. Tetrazolium salt was used for the detection of superoxide radicals and bovine erythrocyte SOD (Cu/Zn) served as standard. Liver and heart (mitochondrial fraction) homogenates, prepared as described in section 2.7.5 and stored at -80°C, were thawed on ice and diluted in sample buffer (50 mM Tris-HCl, pH 8.0) as necessary. Radical detector was added to standards and samples in a 96 well plate and the reaction initiated by adding xanthine oxidase. The 96 well plate was incubated on a shaker for 30 minutes and absorbance read at 450 nm in a FLUOstar Omega Microplate Reader (BMG Labtech, Offenburg, Germany). All samples were assayed in triplicate and results expressed as U/mg protein.

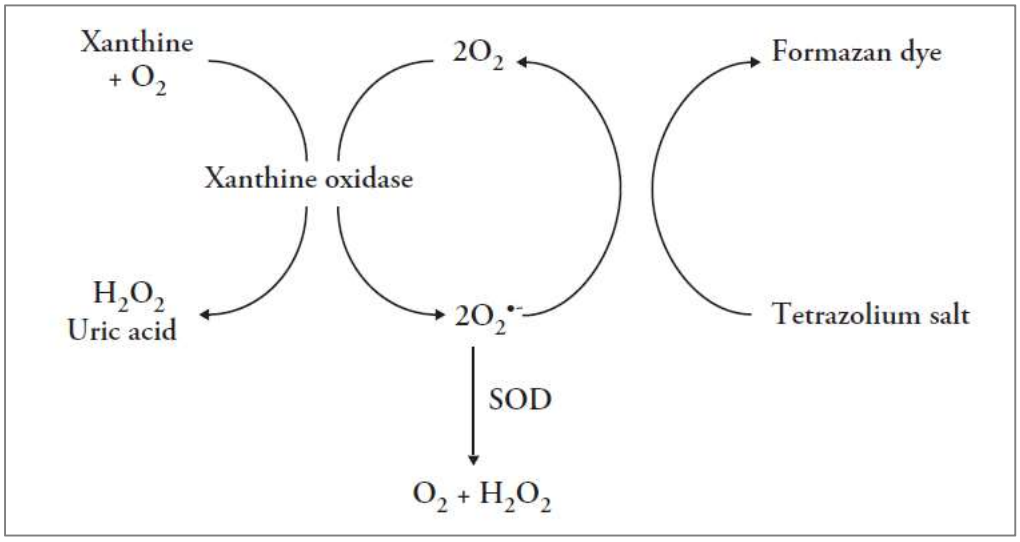

Figure 2.18. Scheme of the Superoxide Dismutase Assay (Cayman Chemical Company, Kit insert).

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2.7.7.2. Catalase

Catalase (CAT) is an ubiquitous antioxidant enzyme present in most aerobic cells and is involved in the detoxification of H_2O_2 by catalysing the conversion of two H_2O_2 molecules to oxygen and two molecules of water. The protocol by Ellerby and Bredesen was adapted for use in a 96 well plate (Ellerby and Bredesen, 2000). In a 96 well clear UV plate, 5 µl diluted sample and 170 µl buffer (50 mM potassium phosphate, pH 7.0) was added and 0.1% H_2O_2 added to initiate the reaction. The linear decline in absorbance was monitored at 240 nm every 30 seconds for five minutes in a FLUOstar Omega Microplate Reader (BMG Labtech, Offenburg, Germany). Liver and heart (cytosolic fraction) homogenates, prepared as described in section 2.7.5 and stored at -80°C, were thawed on ice. Each sample was assayed in triplicate and catalase activity (µmole/min/µg protein) determined using the molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

2.7.7.3. Glutathione Peroxidase

Glutathione peroxidase (GPx) protects cells from oxidative damage by catalysing the reduction of hydroperoxidases (including H_2O_2) by reduced glutathione. The Glutathione Peroxidase Assay Kit (703102) by Cayman Chemical Company (Ann Arbor, MI, USA) was used to measure GPx activity. The assay kit measured the enzymatic activity indirectly by a coupled reaction with glutathione reductase (GR). The oxidised glutathione (GSSG) produced by reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH, according to the reactions in figure 2.19.

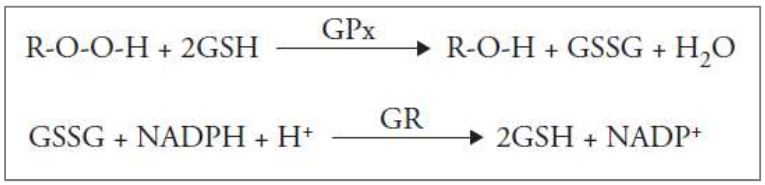

Figure 2.19. Reactions used to determine GPx activity in the Cayman Chemical Company Assay Kit.

Heart (cytosolic fraction) homogenates, prepared as described in section 2.7.5 and stored at -80°C, were thawed on ice. All samples were diluted in sample buffer (50 mM Tris-HCl, pH 7.6) as necessary and assayed in triplicate in a 96 well plate and the decrease in absorbance at 340 nm was read in a FLUOstar Omega Microplate Reader (BMG Labtech, Offenburg,

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Germany) over five minutes and bovine erythrocyte GPx served as positive control. Background wells were free from bovine erythrocyte GPx or sample. GPx activity (µmole/min/mg protein) was calculated using the extinction coefficient of 0.00622 μM^{-1} cm^{-1}.

2.7.7.4. Lipid Peroxidation

Concentrations of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) were kindly analysed by Dr D Blackhurst (Division of Chemical Pathology, University of Cape Town, Cape Town, South Africa). CD and TBARS were measured by spectrophotometric methods using a Labsystems Multiskan MS Analyser (AEC Amersham Co., South Africa). Concentrations were calculated using the appropriate molar extinction coefficients and standards. CD were measured at 234 nm after appropriate dilution in cyclohexane (Spectrosol) as previously described (Esterbauer *et al*., 1989). After mixing the serum samples with cyclohexane, separation was enhanced by centrifugation (14000 × g for 10 min at 10°C). Values were expressed in µmol/l of serum.

Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for the determination of lipid peroxidation, which serves as an indicator of oxidative stress and were measured according to the method of Jentzsch *et al* (1996). Samples (200 µl) were mixed with 10 µl butylated hydroxytoluene (BHT) (Fluka Chemie, Switzerland) in ethanol (Merck Chemicals, South Africa) and orthophosphoric acid (Sigma) buffer at pH 3.6 and vortexed. TBA (Sigma) reagent (25 µl) was added and vortexed again. After incubation at 90°C for 45 minutes in a water bath, the reaction was terminated by placing tubes on ice. TBARS were extracted with n-butanol, saturated NaCl (50 µl) added and the mixture centrifuged at 12000 rpm for 1 minute. Absorbance was read at 532 nm and values were expressed in µmol/l of serum.

Alternatively, the TBARS Assay Kit (10009055) by Cayman Chemical Company (Ann Arbor, MI, USA) was used in our own laboratories to monitor lipid peroxidation in serum samples. Malondialdehyde (MDA) is a product of lipid peroxidation and forms an adduct with thiobarbituric acid (TBA) under high temperatures (90-100°C) and acidic conditions (Fig 2.20). The MDA-TBA adduct was measured colourimetrically at 535 nm in a FLUOstar Omega Microplate Reader (BMG Labtech, Offenburg, Germany).

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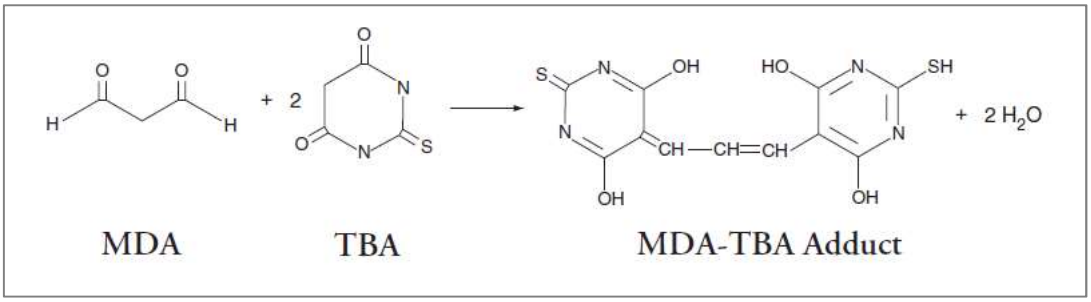

Figure 2.20. Formation of the MDA-TBA Adduct under high temperatures and acidic conditions in the Cayman Chemical Company TBARS Assay Kit.

2.7.8. Inflammatory Markers

Rat serum samples were analysed for TNF-α, IL-6 and CRP on the Bio-Rad Bio-Plex 200 (Hercules, CA, USA) instrument by the Centre for Proteomic & Genomic Research (CPGR, Cape Town, South Africa). Samples were assayed using the R&D Systems™ (Minneapolis, MN, USA) Rat Premixed Multi-Analyte Kit for TNF-α and IL-6 and the ProcartaPlex™ (eBioscience, San Diego, CA, USA) Rat Multiplex Immunoassay kit for CRP. Cytokine data was analysed using the Bio-Plex Manager 6.1 software. Three multi-analyte plates (for TNF-α and IL-6) and CRP simplex plates were run to cover all serum samples and a standard curve was generated for each analyte on each plate. All standard curves were optimized by the software by identifying outliers that demonstrate poor standard curve recovery, poor intra-assay percentage coefficient of variation (%CV) and saturation.

2.7.9. Serum Lipids

Serum total cholesterol (TC), triglycerides (TG) and phospholipid (PL) concentrations were kindly analysed by Dr D Blackhurst (Division of Chemical Pathology, University of Cape Town, Cape Town, South Africa). TC, TG and PL concentrations were determined by using enzymatic colorimetric kits (WAKO Chemicals, Germany) with a Labsystems Multiskan MS analyzer (AEC Amersham Co., South Africa). TC were determined by pipetting 3 µl of the blank (saline), standard or sample into wells of a 96 well plate and adding 300 µl of colour solution to each well. After mixing, the plate was incubated for five minutes at 37°C and absorbance read at 600 nm. TG were determined by pipetting 4 µl blank (saline), standard (multi-lipid calibrator) or sample into the wells of a 96 well plate and adding 90 µl of Colour A. After mixing, the plate was incubated at 37°C for five minutes and absorbance measured at 600 nm. This measurement served as sample blank. Following initial measurement, 30 µl of

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Colour B was added to each well. After mixing, the plate was incubated at 37°C for five minutes and absorbance measured at 600 nm. Final absorbance was determined by subtracting the first measurement from the second measurement. The TG concentration of samples was determined using the multi-lipid calibrator's concentration and absorbance values from the standard (calibration) curve. Phospholipids were determined by pipetting 3 µl blank (deiH_2O), standard or sample into the wells of a 96 well plate and adding 300 µl Colour reagent solution to each well. After mixing, the plate was incubated at 37°C for 10 minutes and absorbance measured at 600 nm. Values for TC, TG and PL were expressed in mmol/l of serum.

2.7.10. Statistical Analysis

Data were analysed using GraphPad Prism® 5 software (GraphPad Software, San Diego, CA, USA). Data were statistically analysed by means of one-way analysis of variance, followed by the Bonferroni post-test. Differences with a p-value < 0.05 were considered statistically significant. A statistician at the Biostatistics Unit, Centre for Evidence Based Health Care, Faculty of Medicine and Health Sciences, Stellenbosch University, was consulted.

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CHAPTER 3: RESULTS

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3.1. Soluble Solids, Total Polyphenols and Flavonoid Content Determinations of Rooibos

Unfermented rooibos had a significantly higher soluble solid content and total polyphenolic content than fermented rooibos (Soluble solids: RUF 4.6 ± 0.40 mg/ml; RF 3.5 ± 0.22 mg/ml, total polyphenolic content: RUF 0.23 ± 0.03 mg gallic acid/mg soluble solids; RF 0.16 ± 0.01 mg gallic acid/mg soluble solids). The daily total phenolic intake of the unfermented rooibos treatment groups (2% RUF and 2% RUF and 5 mg/kg bw/day nicotine co-treatment) were also significantly higher than that of the fermented rooibos treatment groups (2% RF and 2% RF and 5 mg/kg bw/day nicotine co-treatment) (Table 3.1).

Fermented rooibos had a significantly higher flavonol content (0.36 ± 0.02 mg quercetin equivalents/mg soluble solids) than unfermented rooibos (0.18 ± 0.02 mg quercetin equivalents/mg soluble solids). The daily flavonol intake of the fermented rooibos treatment groups were also significantly higher than that of the unfermented rooibos treatment groups (Table 3.2). Unfermented rooibos had a significantly higher flavanol content (0.10 ± 0.01 mg catechin equivalents/mg soluble solids) than fermented rooibos (0.05 ± 0.00 mg catechin equivalents/mg soluble solids). The daily flavanol intake of the unfermented rooibos treatment groups were also significantly higher than that of the fermented rooibos treatment groups (Table 3.3).

Values of known flavonoid compounds, as determined by HPLC analysis, is given in table 3.4 (fermented rooibos) and table 3.5 (unfermented rooibos).

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Table 3.1. Soluble solid and total polyphenolic content of 2% fermented rooibos and 2% unfermented rooibos.

	Soluble solids (mg/ml)	**Total phenolic content (mg gallic acid/mg soluble solids)**	**Daily total phenolic intake (mg gallic acid equivalents/ day / 100g BW)**		**Daily total phenolic intake (mg gallic acid equivalents/ day / 100g BW)**
2% RF	3.5 ± 0.22	0.16 ± 0.01	5.17 ± 0.28	**NRF**	4.86 ± 0.31
2% RUF	4.6 ± 0.40#	0.23 ± 0.03#	9.43 ± 0.46#	**NRUF**	8.07 ± 0.26#

BW (Body Weight); NRF (Nicotine 5 mg/kg bw/day + 2% Fermented Rooibos Co-treatment); NRUF (Nicotine 5 mg/kg bw/day + 2% Unfermented Rooibos Co-treatment); # p<0.05 vs 2% fermented rooibos treatment groups; n = 6.

Table 3.2. Flavonol content of 2% fermented and 2% unfermented rooibos.

	Flavonol content (mg quercetin equivalents/mg soluble solids)	**Daily flavonol intake (mg quercetin equivalents/ day/ 100g BW)**		**Daily flavonol intake (mg quercetin equivalents/ day/ 100g BW)**
2% RF	0.36 ± 0.02@	1.11 ± 0.06@	**NRF**	1.04 ± 0.07@
2% RUF	0.18 ± 0.02	0.74 ± 0.04	**NRUF**	0.63 ± 0.02

BW (Body Weight); NRF (Nicotine 5 mg/kg bw/day + 2% Fermented Rooibos Co-treatment); NRUF (Nicotine 5 mg/kg bw/day + 2% Unfermented Rooibos Co-treatment); @ p<0.05 vs 2% unfermented rooibos treatment groups; n = 6.

Table 3.3. Flavanol content of 2% fermented and 2% unfermented rooibos.

	Flavanol content (mg catechin equivalents/mg soluble solids)	**Daily flavanol intake (mg catechin equivalents/ day/ 100g BW)**		**Daily flavanol intake (mg catechin equivalents/ day/ 100g BW)**
2% RF	0.05 ± 0.00	0.10 ± 0.01	**NRF**	0.09 ± 0.01
2% RUF	0.10 ± 0.01#	0.37 ± 0.02#	**NRUF**	0.32 ± 0.01#

BW (Body Weight); NRF (Nicotine 5 mg/kg bw/day + 2% Fermented Rooibos Co-treatment); NRUF (Nicotine 5 mg/kg bw/day + 2% Unfermented Rooibos Co-treatment); # p<0.05 vs 2% fermented rooibos treatment groups; n = 6.

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Table 3.4. HPLC quantification of flavonoids in 2% fermented rooibos consumed by rats.

	2% RF		
	Concentration (µg/ml)	% of Soluble Solids	Daily intake (mg/100 g BW)
Phenylpyruvic acid-2-O-glucoside (PPAG)	13.42 ± 0.00	0.391 ± 0.03	0.124 ± 0.01
Aspalathin	7.57 ± 0.00	0.221 ± 0.01	0.070 ± 0.01
Nothofagin	1.76 ± 0.00	0.051 ± 0.01	0.016 ± 0.00
Isoorientin	31.99 ± 0.00	0.933 ± 0.06	0.295 ± 0.02
Orientin	28.86 ± 0.00	0.842 ± 0.05	0.266 ± 0.01
Ferulic acid	1.87 ± 0.00	0.055 ± 0.01	0.017 ± 0.00
Quercetin-3-robinobioside	19.27 ± 0.00	0.562 ± 0.04	0.178 ± 0.01
Vitexin	5.65 ± 0.00	0.165 ± 0.01	0.052 ± 0.01
Hyperoside	5.40 ± 0.00	0.156 ± 0.01	0.050 ± 0.01
Rutin	1.60 ± 0.00	0.047 ± 0.01	0.015 ± 0.00
Isovitexin	5.76 ± 0.00	0.168 ± 0.01	0.053 ± 0.01
Isoquercitrin	3.64 ± 0.00	0.106 ± 0.01	0.034 ± 0.01
Luteolin-7-glucoside	0.83 ± 0.00	0.024 ± 0.01	0.008 ± 0.00

BW (body weight), RF (fermented rooibos treatment group); n = 5-6.

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Table 3.5. HPLC quantification of flavonoids in 2% unfermented rooibos consumed by rats.

	2% RUF		
	Concentration (µg/ml)	% of Soluble Solids	Daily intake (mg/100 g BW)
PPAG	15.93 ± 0.00	0.361 ± 0.04	0.148 ± 0.01
Aspalathin	392.96 ± 0.00	8.907 ± 1.05	3.645 ± 0.18
Nothofagin	57.85 ± 0.00	1.311 ± 0.15	0.537 ± 0.03
Isoorientin	65.22 ± 0.00	1.478 ± 0.17	0.605 ± 0.03
Orientin	49.95 ± 0.00	1.132 ± 0.13	0.463 ± 0.02
Ferulic acid	not detected		
Quercetin-3-robinobioside	17.46 ± 0.00	0.395 ± 0.05	0.162 ± 0.01
Vitexin	7.42 ± 0.00	0.168 ± 0.02	0.069 ± 0.01
Hyperoside	2.92 ± 0.00	0.066 ± 0.01	0.027 ± 0.01
Rutin	13.82 ± 0.00	0.313 ± 0.04	0.128 ± 0.01
Isovitexin	9.88 ± 0.00	0.224 ± 0.03	0.091 ± 0.01
Isoquercitrin	4.40 ± 0.00	0.099 ± 0.01	0.041 ± 0.01
Luteolin-7-glucoside	1.40 ± 0.00	0.031 ± 0.01	0.013 ± 0.00

PPAG (Phenylpyruvic acid-2-O-glucoside), BW (body weight), RUF (unfermented rooibos treatment group); n = 5-6.

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3.2. *In vivo* and *Ex vivo* Investigations: Pilot Study

3.2.1. Introduction

Before the main *in vivo* and *ex vivo* studies were undertaken, an optimal vascular injury-inducing concentration of nicotine had to be established in our experimental setting. Based on literature, 1 mg/kg bw/day and 2 mg/kg bw/day (Benowitz and Jacob, 1999; Balakumar *et al*., 2008b) nicotine were chosen for the concentration-response pilot studies, and compared to saline vehicle control over a six week treatment period. Nicotine and saline were administered via the subcutaneous route.

3.2.2. Specific Aims

The pilot study aimed to determine an optimal vascular injury-inducing concentration in an *in vivo* Wistar rat model by assessment of the following endpoints:

3.2.2.1. Biometric Measurements and Fluid Intake

Biometric data, including start and end body weights, as well as weekly fluid intakes were recorded.

3.2.2.2. Blood Pressure

Blood pressure was non-invasively measured by means of the CODA^{TM} tail-cuff method.

3.2.2.3. Vascular Function

Vascular relaxation responses were determined in aortic rings by means of isometric tension studies.

3.2.2.4. Lipid Peroxidation

Lipid peroxidation was measured in serum samples by means of the TBARS assay.

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3.2.3. Results

3.2.3.1. Biometric Measurements and Fluid Intake

Nicotine treatments did not result in a significant difference in the mean weight gain when compared to saline controls (vehicle control: 55.83 ± 4.19 g; 1 mg/kg bw/day nicotine: 55 ± 3.62 g; 2 mg/kg bw/day 41.68 ± 9.52 g) (Fig 3.1). Nicotine resulted in a significant decrease in mean daily fluid intake in both 1 mg/kg bw/day and 2 mg/kg bw/day when compared to saline vehicle control (vehicle control: 32.43 ± 1.452 ml; 1 mg/kg bw/day nicotine: 26 ± 0.71 ml; 2 mg/kg bw/day nicotine 23.89 ± 0.78 ml) (Fig 3.2).

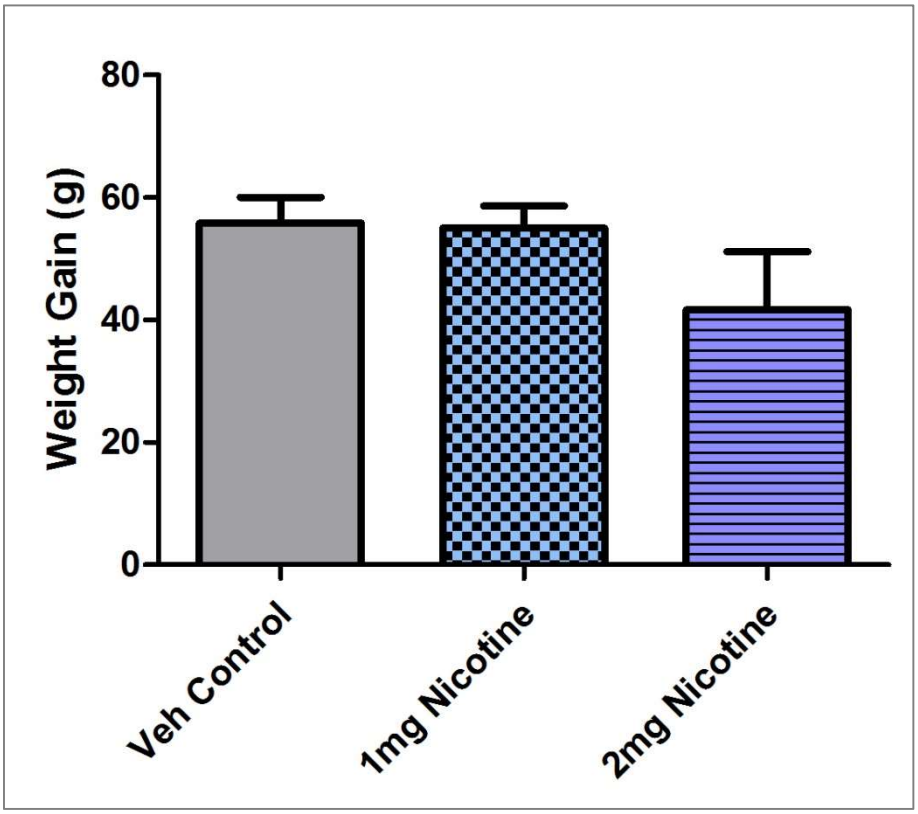

Figure 3.1. Mean weight gain over the six week treatment period. No significant differences were found (n = 5 per group).

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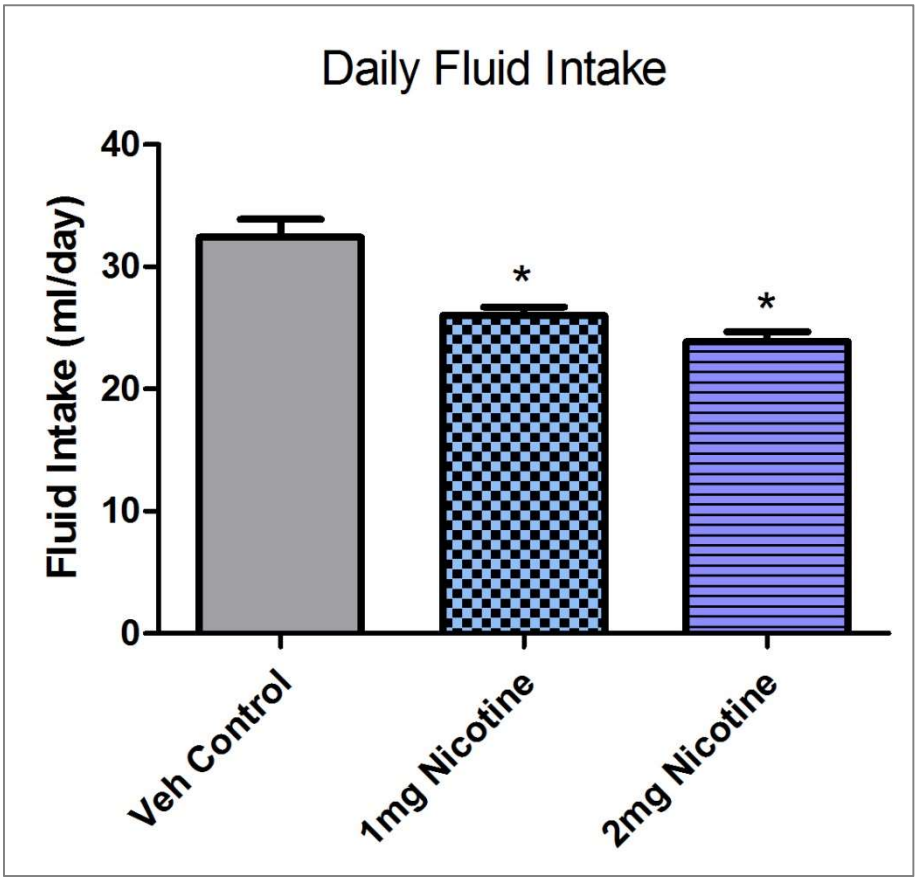

Figure 3.2. Mean daily fluid intake over the six week treatment period. Both 1 mg/kg bw/day and 2 mg/kg bw/day nicotine treatment resulted in significantly lower fluid (water) intake when compared to the saline vehicle control group. * $p<0.05$ vs control (n = 5 per group).

3.2.3.2. Blood Pressure

Blood pressure measurements by means of the non-invasive tail-cuff method did not demonstrate any significant changes at the end of the six week treatment period in mean systolic (Fig 3.3 A), mean diastolic (Fig 3.3 B) or mean arterial blood pressure (Fig 3.3 C) in 1 mg/kg bw/day or 2 mg/kg bw/day nicotine treatment groups when compared to the saline vehicle control group (Table 3.6).

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Table 3.6. Mean systolic, mean diastolic and mean arterial blood pressure in the saline vehicle control, 1mg/kg bw/day and 2mg/kg bw/day nicotine treated groups.

	Mean Systolic Pressure	Mean Diastolic Pressure	Mean Arterial Pressure
Saline Vehicle Control	161.10 ± 7.28 mmHg	118.10 ± 6.76 mmHg	134.20 ± 4.96 mmHg
1 mg/kg bw/day nicotine	147.10 ± 8.27 mmHg	105.90 ± 8.20 mmHg	120.70 ± 8.64 mmHg
2 mg/kg bw/day nicotine	148.30 ±9.37 mmHg	113.40 ± 10.25 mmHg	124.70 ± 9.93 mmHg

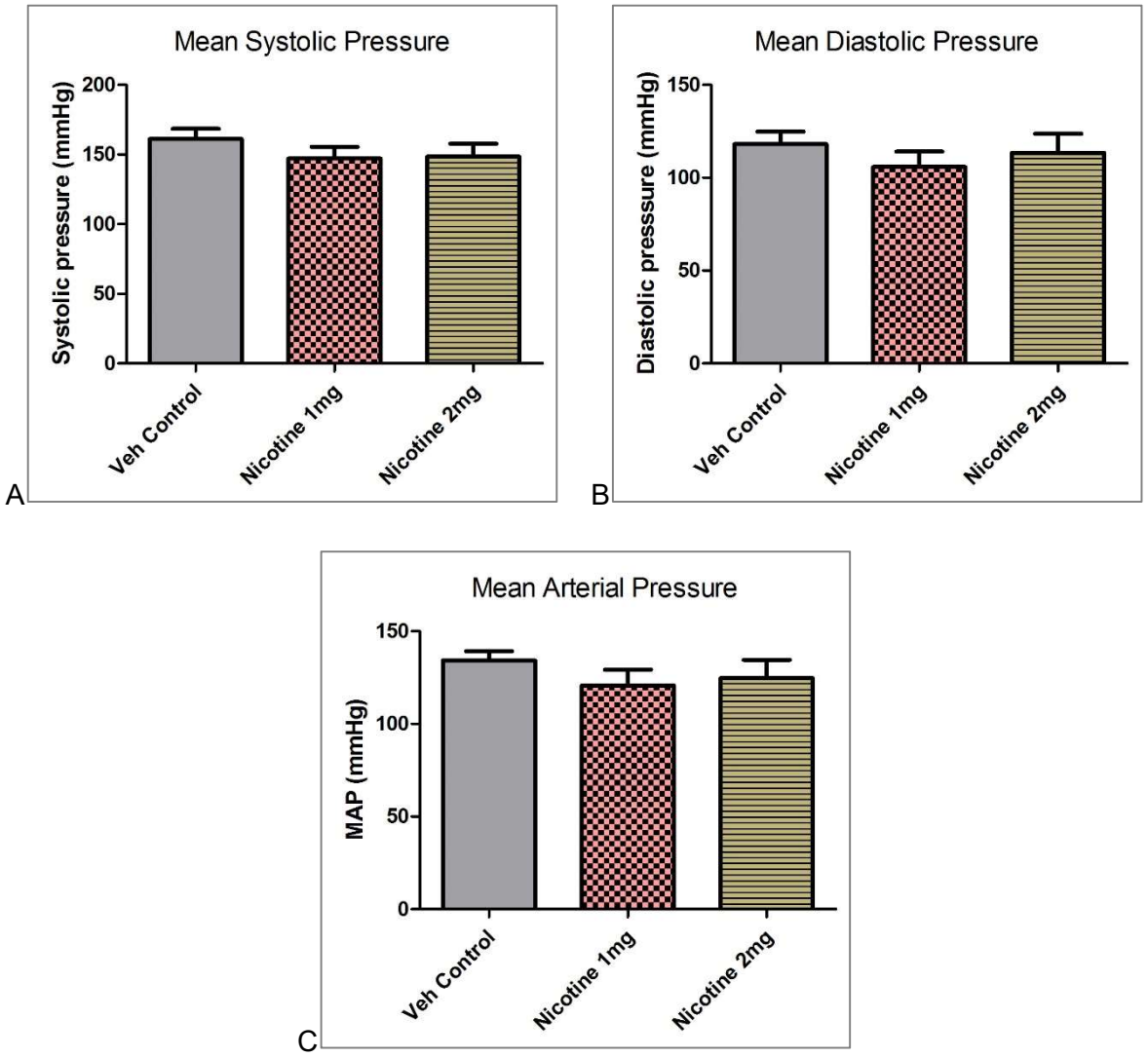

Figure 3.3. The effects of administration of 1 mg/kg bw/day and 2 mg/kg bw/day nicotine on blood pressure over a six week treatment period. The administration of 1 mg/kg bw/day and 2 mg/kg bw/day nicotine resulted in no significant changes in A) mean systolic blood pressure; B) mean diastolic blood pressure and C) mean arterial pressure when compared to saline vehicle control rats (n = 5 per group).

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3.2.3.3. Vascular Function by means of Isometric Tension Studies in Aortic Rings

Vascular function was determined by means of isometric tension studies in aortic rings. After contraction was induced by 100 nM Phe, ACh was added in a cumulative manner in order to determine endothelium-dependent relaxation. Cumulative ACh relaxation demonstrated no significant differences between the 1 mg/kg bw/day and 2 mg/kg bw/day nicotine treatment groups, or the saline vehicle control group (Fig 3.4).

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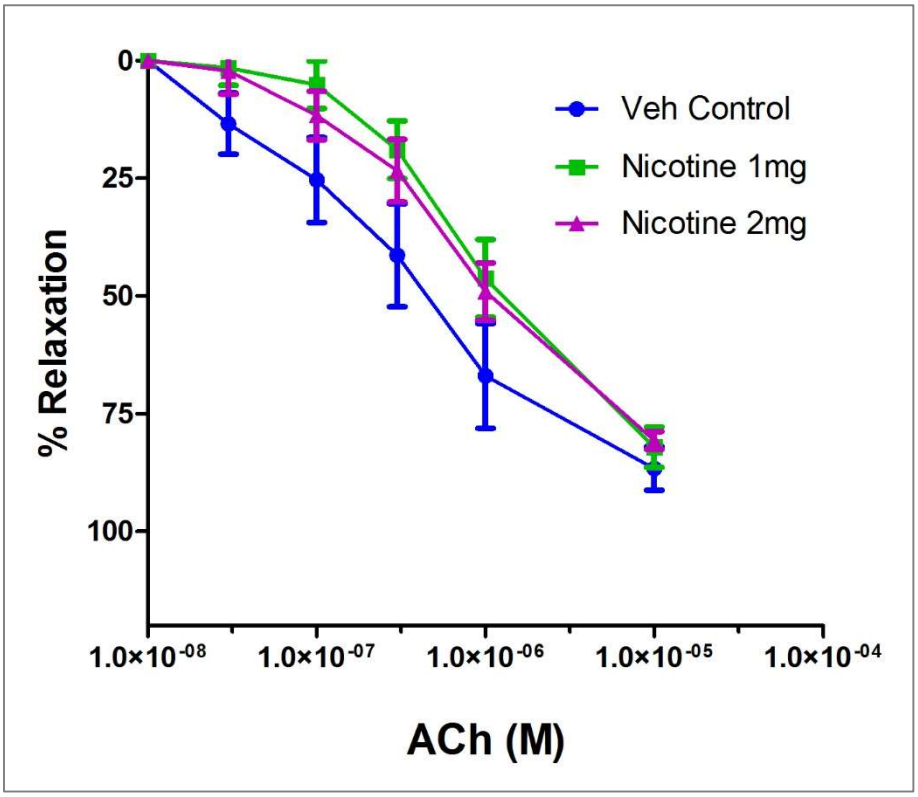

Figure 3.4. The administration of 1 mg/kg bw/day and 2 mg/kg bw/day nicotine over a six week treatment period did not result in significant effects on aortic ring relaxation when compared to saline vehicle control aortic rings (n = 4-5 per group).

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3.2.3.4. Lipid Peroxidation: TBARS

Lipid peroxidation measurements, as determined by a standard TBARS assay kit showed no significant differences in MDA equivalents in the 1 mg/kg bw/day and 2 mg/kg bw/day nicotine treatment groups as compared to the vehicle control group (vehicle control: 6.95 ± 0.60 µM; 1 mg/kg bw/day nicotine: 5.59 ± 0.71 µM; 2 mg/kg bw/day nicotine 5.93 ± 0.27 µM) (Fig 3.5).

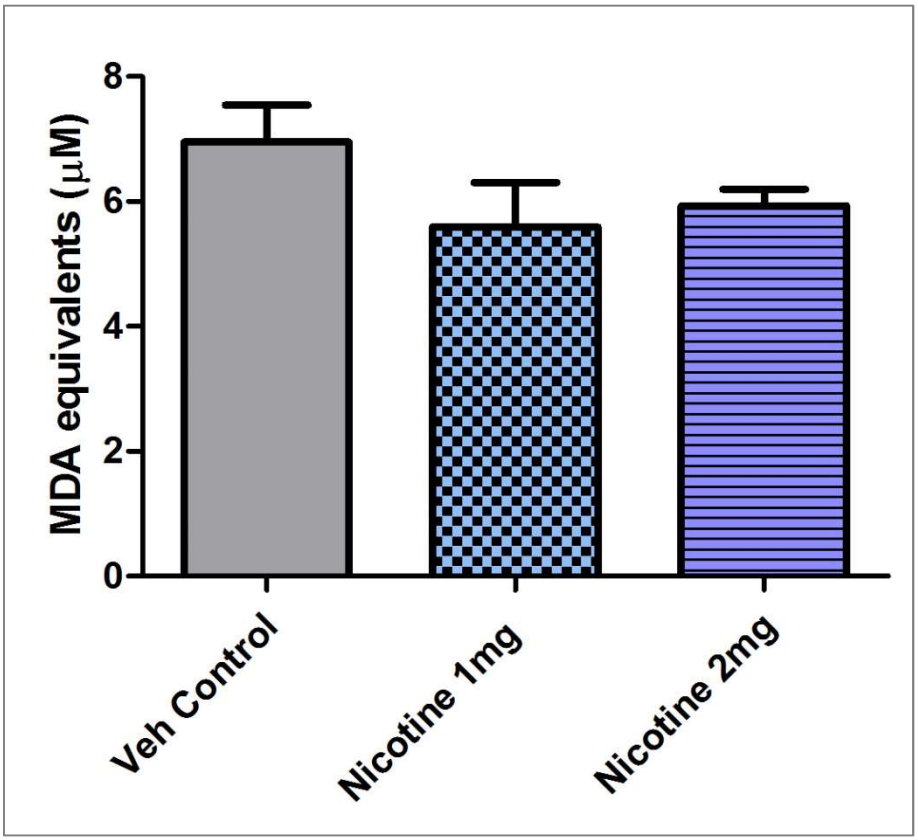

Figure 3.5. There were no significant differences in serum lipid peroxidation measurements (TBARS assay) between 1 mg/kg bw/day and 2 mg/kg bw/day nicotine treated rats as compared to saline vehicle control rats (n = 5 per group).

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3.2.4. Summary of Pilot Study Results

A six week treatment period with 1 mg/kg bw/day and 2 mg/kg bw/day nicotine did not result in any significant differences in blood pressure, vascular function or lipid peroxidation when compared to the saline vehicle control. Mean body weights at the end of the treatment period were not significantly influenced. Mean daily fluid intake was the only endpoint that was significantly affected by treatment with 1 mg/kg bw/day and 2 mg/kg bw/day nicotine. Administration of nicotine at both concentrations resulted in a significant reduction in fluid intake when compared to the saline vehicle control.

Based on these results, it was concluded that six weeks treatment with 1 mg/kg bw/day or 2 mg/kg bw/day nicotine did not induce sufficient injury in our experimental setting. As a result of these findings, it was decided to perform the main study by treating the rats with a higher nicotine concentration (5 mg/kg bw/day) as previously used in another study (Zimmerman and McGeachie, 1985).

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3.3. *In vivo* and *Ex vivo* Investigations: Main Study

3.3.1. Introduction

The modulating capabilities of rooibos (fermented and unfermented) and melatonin in a model of nicotine-induced vascular injury was assessed *in vivo* and ex *vivo* according to the specific aims listed in section 3.3.2.

3.3.2. Specific Aims

3.3.2.1. Biometric Measurements and Fluid Intake

Biometric measurements, including mean body weights, mean organ weights (heart and liver), as well as the mean weekly fluid intake were recorded.

3.3.2.2. Blood Pressure

Blood pressure was measured non-invasively by means of the CODA^{TM} tail-cuff method.

3.3.2.3. Vascular Function

Vascular contraction / relaxation responses were determined in aortic rings by means of isometric tension studies. In a separate set of experiments, the cellular effects of nicotine and rooibos were further investigated utilising a cultured rat aortic endothelial cell model.

3.3.2.4. Antioxidant Enzyme Activity and Oxidative Stress Status

The antioxidant enxyme activity and oxidative stress status was measured by determining SOD activity and CAT activity in heart and liver tissue homogenates and GPx activity in heart tissue homogenates. Lipid peroxidation was determined in serum samples.

3.3.2.5. Inflammatory Markers

Serum samples were analysed for TNF-α, IL-6 and CRP levels.

3.3.2.6. Serum Lipid Measurements

Total cholesterol, triglycerides and phospholipid concentrations were determined in serum samples.

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3.3.3. Results

For the main *in vivo* and *ex vivo* study a total of 90 rats were divided into nine groups (n = 10 per group), namely nicotine and saline vehicle control, as well as nicotine co-treated with either fermented rooibos, unfermented rooibos or melatonin, and groups receiving water, rooibos (fermented or unfermented) or melatonin. The groups received the respective treatments for six weeks and the following data were obtained. The abbreviations defined in Table 3.7 are frequently used to describe treatment groups.

Table 3.7. Abbreviations used for treatment groups

Abbreviation	**Treatment Group**
Veh Control	Saline Vehicle Control (subcutaneous administration) for nicotine treated groups (nicotine, NRF, NRUF, NMel)
Water Control	Drinking Control (tap water) for rooibos and melatonin groups
Nicotine	Nicotine 5 mg/kg bw/day (subcutaneous administration)
RF	Rooibos Fermented 2%
RUF	Rooibos Unfermented 2%
Mel	Melatonin 4 mg/kg bw/day
NRF	Nicotine 5 mg/kg bw/day + Rooibos Fermented 2%
NRUF	Nicotine 5 mg/kg bw/day + Rooibos Unfermented 2%
NMel	Nicotine 5 mg/kg bw/day + Melatonin 4 mg/kg bw/day

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3.3.3.1. Biometric Measurements and Fluid Intake

3.3.3.1.1. Mean Body Weight Gain

The veh control (53.83 ± 10.50 g), water control (93.82 ± 9.97 g), RF (96.4 ± 6.59 g), RUF (93.24 ± 6.97 g) and Mel (56.17 ± 10.32 g) groups showed a significant increase in mean body weight gain over the six week treatment period when compared to the nicotine (4.71 ± 5.68 g), NRF (8.46 ± 3.45 g), NRUF (18.09 ± 8.89 g) and NMel (-7.28 ± 5.35 g) treatment groups. In addition, the water control, RF and RUF groups showed a significant increase in weight gain over the six week treatment period when compared to the veh control and Mel groups (Fig 3.6).

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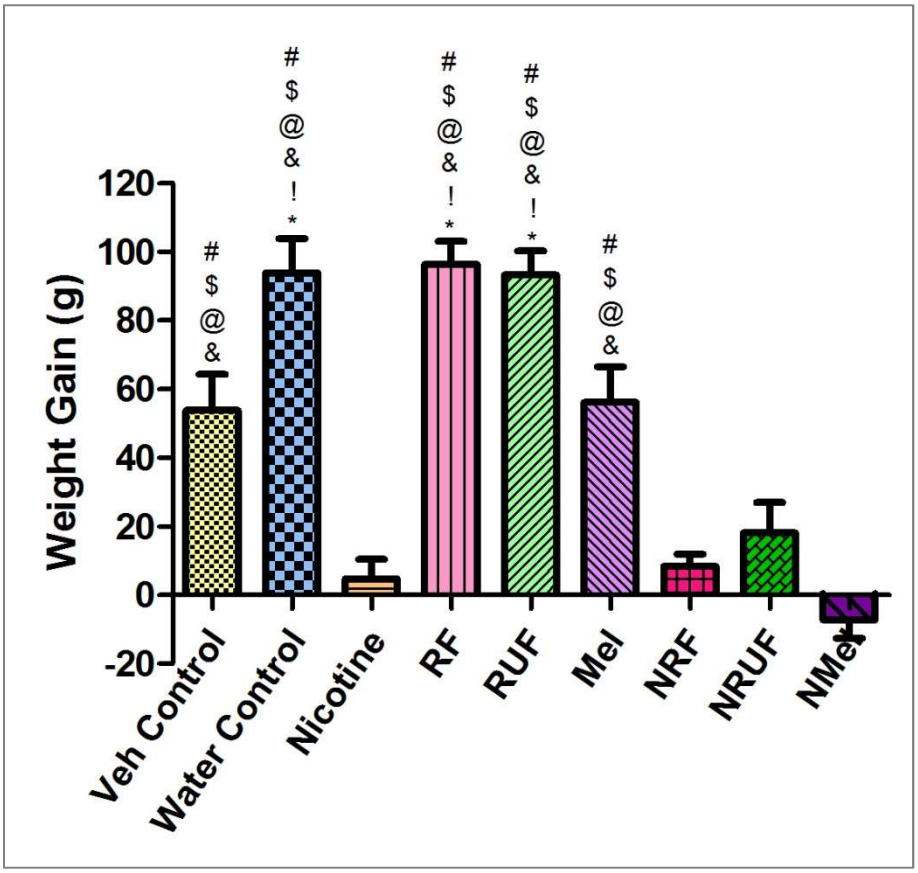

Figure 3.6. Mean weight gain at the end of the six week treatment period. # p<0.05 vs nicotine; $ p< 0.05 vs NRF; @ p<0.05 vs NRUF; & p<0.05 vs NMel; ! p<0.05 vs veh control; * p<0.05 vs Mel (n = 10 per group).

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3.3.3.1.2. Mean Relative Organ Weights

The ratio of the mean heart weight/body weight and mean liver weight/body weight were determined for all treatment groups. The mean heart weight/body weight ratio was significantly increased in the nicotine group (0.308 ± 0.01) when compared to the water control (0.266 ± 0.006), RF (0.273 ± 0.01) and NMel (0.272 ± 0.01) groups; however, there was no difference compared to the veh control group. The mean heart weight/body weight ratio was also significantly increased in the NRUF group (0.300 ± 0.01) when compared to the water control group (Fig 3.7).

The mean liver weight/body weight ratio was significantly increased in the nicotine group (3.368 ± 0.06) when compared to the water control (2.943 ± 0.06), RF (2.941 ± 0.04), RUF (2.865 ± 0.06), Mel (2.789 ± 0.07), NRF (3.010 ± 0.08), NRUF (3.003 ± 0.07) and NMel groups (2.907 ± 0.07); however, there was no difference between the nicotine group and its veh control group. The mean liver weight/body weight ratio of the vehicle control group (3.180 ± 0.07) was significantly increased when compared to the RUF and Mel groups.

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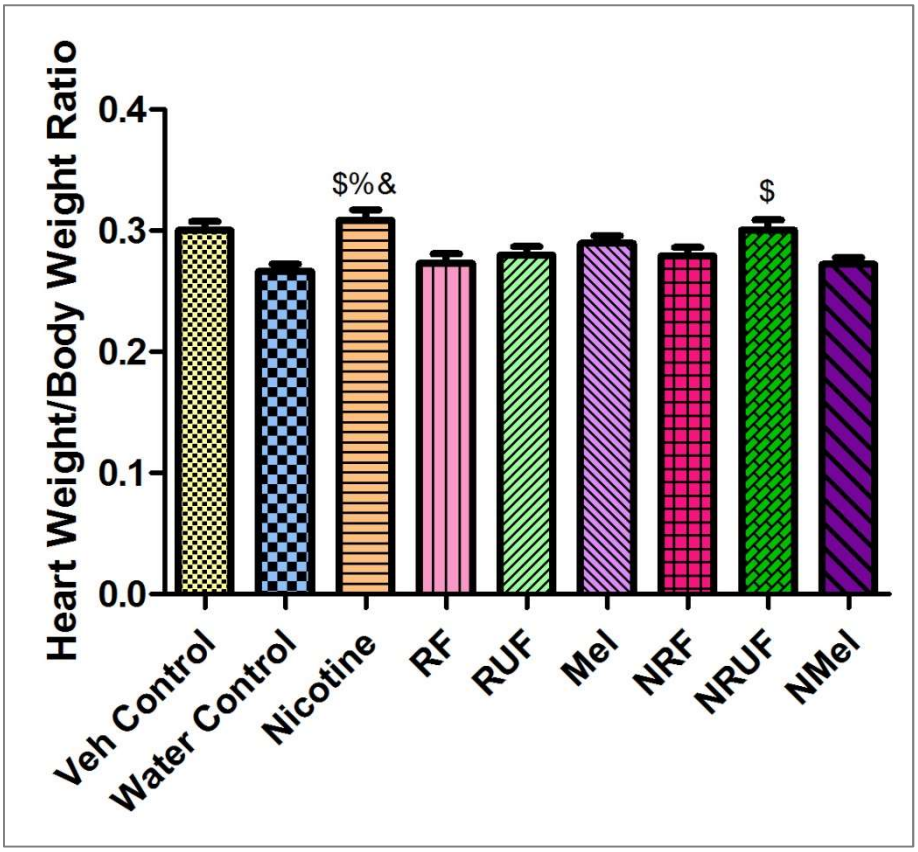

Figure 3.7. Mean heart weight/body weight ratio. $ p<0.05 vs water control; % p<0.05 vs RF; & p<0.05 vs NMel (n = 10 per group).

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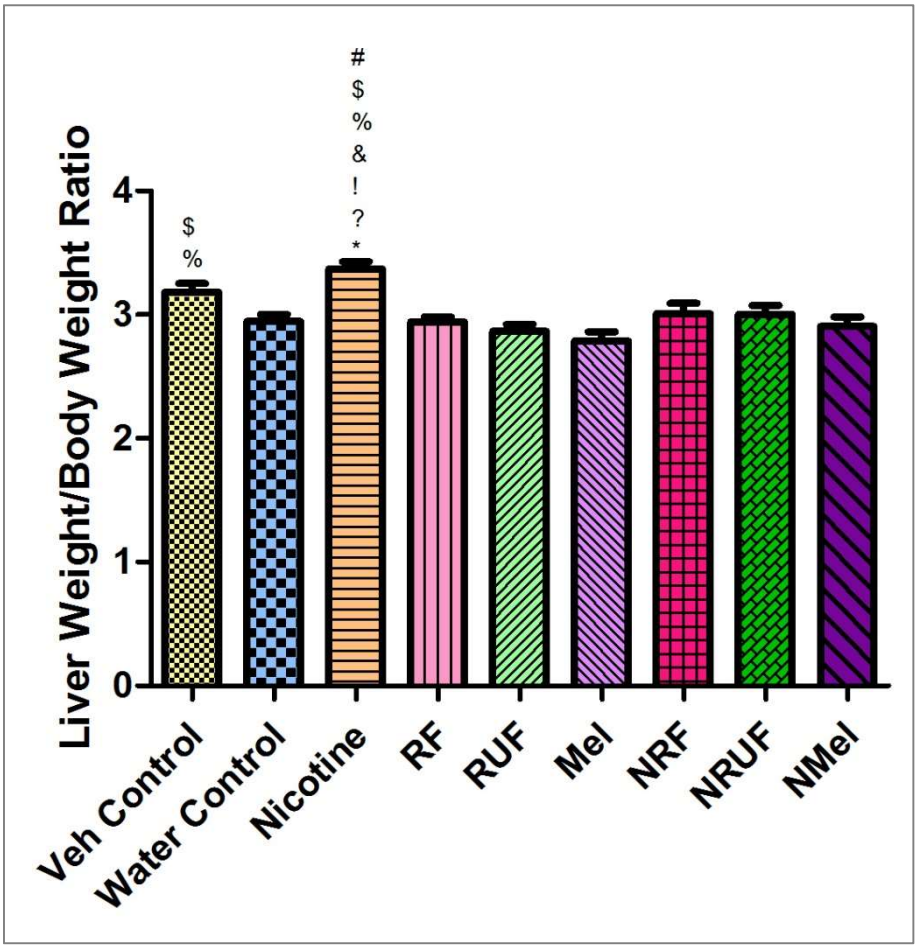

Figure 3.8. Mean liver weight/body weight ratio. $ p<0.05 vs RUF; % p<0.05 vs Mel; # p<0.05 vs RF; * p<0.05 vs water control; & p<0.05 vs NRF; ! p<0.05 vs NRUF; ? p<0.05 vs NMel (n = 10 per group).

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3.3.3.1.3. Fluid Intake

Rats were caged individually and fluid intake was measured weekly (water, fermented rooibos, unfermented rooibos) or daily (melatonin). Figure 3.9 shows the mean daily fluid intake (ml) of each treatment group. The mean daily fluid intake of the water control (26.09 ± 0.81 ml), nicotine (25.38 ± 1.14 ml), NRF (25.33 ± 1.92 ml) and NRUF (23.99 ± 0.87 ml) groups were significantly reduced when compared to the veh control (34.87 ± 2.67 ml), RF (35.17 ± 1.91 ml), RUF (35.81 ± 1.45 ml), Mel (36.63 ± 1.74 ml) and NMel (35.79 ± 1.75 ml) groups.

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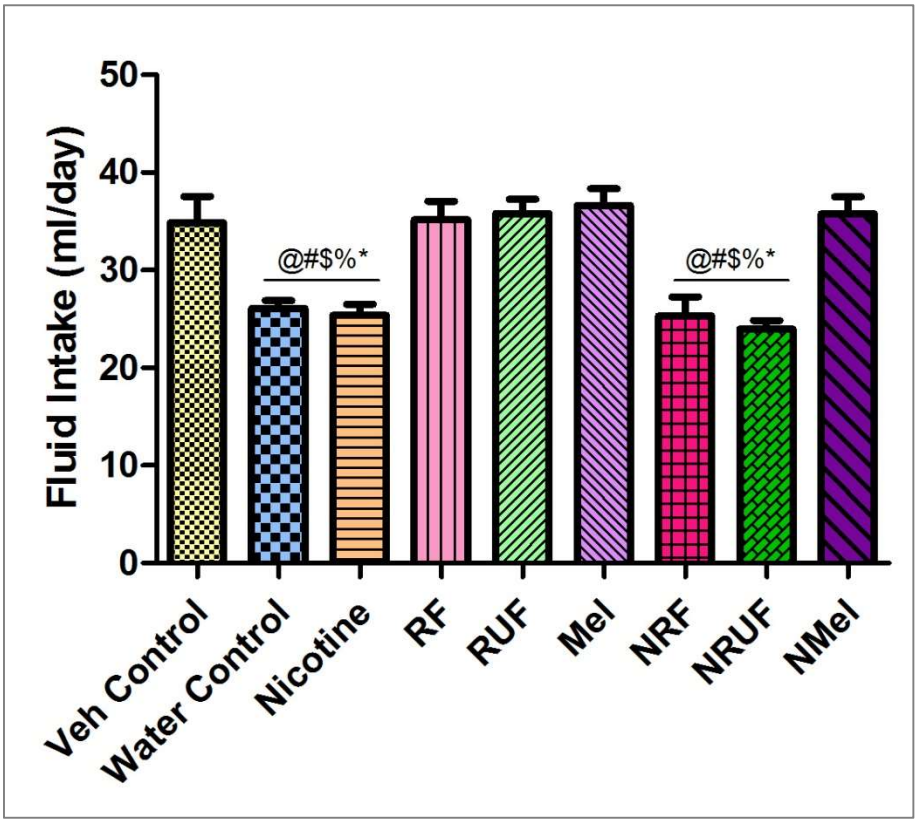

Figure 3.9. Mean daily fluid intakes of all treatment groups. @ p<0.05 vs veh control; # p<0.05 vs RF; $ p<0.05 vs RUF; % p<0.05 vs Mel; * p<0.05 vs NMel (n = 10 per group).

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3.3.3.2. Blood Pressure

Blood pressure was non-invasively measured by means of the CODA^{TM} tail-cuff system. Rats were acclimatised to the apparatus for one week prior to measurements. Results reflect blood pressure at the end of the six week treatment period. However, due to noise levels and interruptions attributed to building works taking place in the Central Animal Housing Unit of the Faculty of Medicine and Health Sciences, blood pressure readings could not be completed for all groups.

In the assessed groups it was found that mean systolic pressure was significantly increased in the water control (145.1 ± 1.48 mmHg), nicotine (145.0 ± 3.02 mmHg) and RUF (147.6 ± 3.54 mmHg) groups when compared to the veh control group (122.4 ± 3.47 mmHg) (Fig 3.10). Nicotine treatment also significantly increased mean diastolic pressure (114.8 ± 1.99 mmHg) when compared to the veh control (100.3 ± 1.93 mmHg) and NMel (84.21 ± 2.28 mmHg) groups and mean arterial pressure (116.3 ± 1.93 mmHg) when compared to the veh control group (100.2 ± 1.83 mmHg) and NMel (100.7 ± 1.80 mmHg) groups (Fig 3.11 and Fig 3.12).

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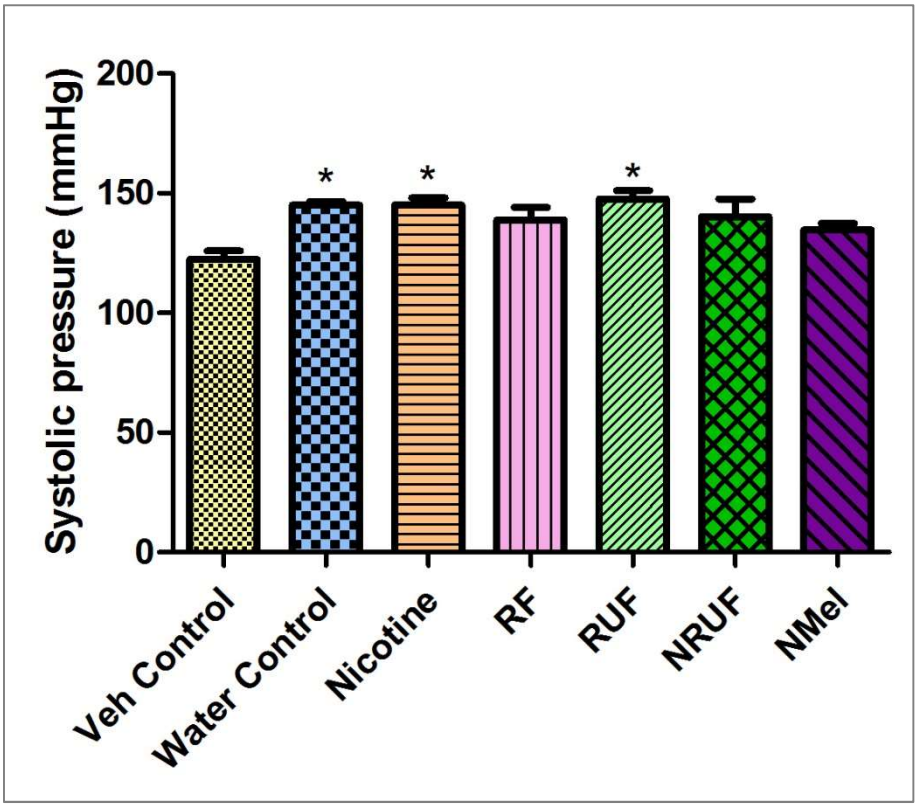

Figure 3.10. Mean systolic blood pressure was significantly increased in the water control, nicotine and RUF groups when compared to the veh control group; * $p<0.05$ vs veh control (n = 4-8 per group).

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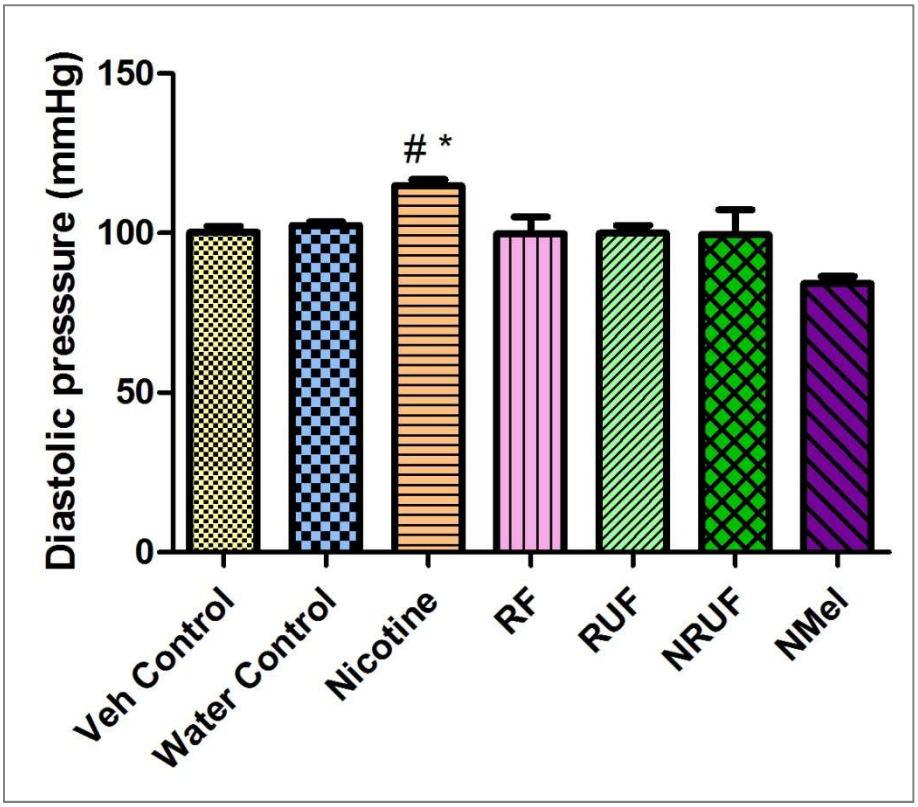

Figure 3.11. Mean diastolic blood pressure was significantly increased in the nicotine group when compared to the veh control and NMel groups; * $p<0.05$ vs veh control; # $p<0.05$ vs NMel (n = 4-8 per group).

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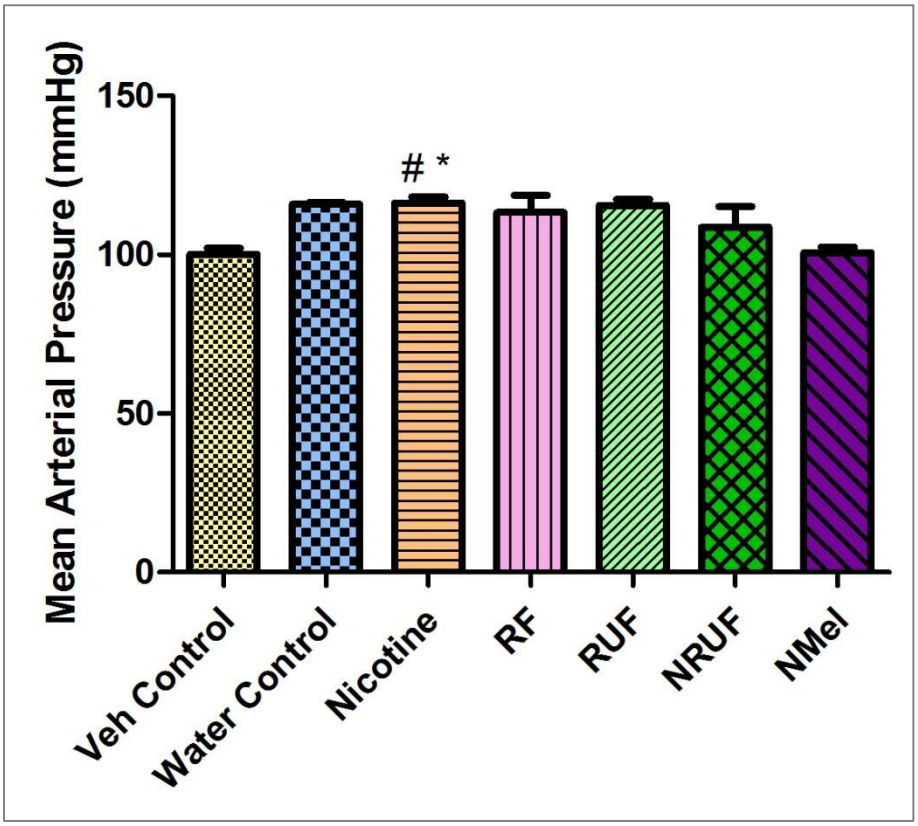

Figure 3.12. The mean arterial pressure in the nicotine group was significantly increased when compared to the veh control and NMel groups; * $p<0.05$ vs vehicle control; # $p<0.05$ vs NMel (n = 4-8 per group).

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3.3.3.3. Vascular Function

3.3.3.3.1. Vascular Contraction / Relaxation Function Measurements by means of Isometric Tension Studies in Aortic Rings

The vascular function of all treatment groups was assessed by means of aortic ring isometric tension studies. The experimental protocol made use of cumulative additions of Phe and ACh to test the functionality of the endothelium.

Aortic rings from the nicotine treatment rats showed a significant pro-contractile response to Phe administration when compared to the saline vehicle control (Fig 3.13), but had no significant effect on relaxation (Fig 3.14). Aortic rings from Mel treatment rats showed a significant anti-contractile response to Phe administration when compared to the water control, RF and RUF treatment groups (Fig 3.15). Aortic rings from Mel, RF and RUF treatment rats showed a significant pro-relaxation response to ACh administration when compared to the water control group (Fig 3.16).

Aortic rings from NMel, NRF and NRUF treatment rats showed a significant anti-contractile response to Phe administration when compared to the nicotine treatment group. Additionally, aortic rings from NMel treatment rats also showed a significant anti-contractile response to Phe administration when compared to the NRF and NRUF treatment groups (Fig 3.17). Aortic rings from NMel and NRF treatment rats showed a significant pro-relaxation response to Ach administration when compared to the nicotine and NRUF groups. Aortic rings from the NMel treatment rats also showed a significant pro-relaxation response to ACh administration when compared to the NRUF group (Fig 3.18).

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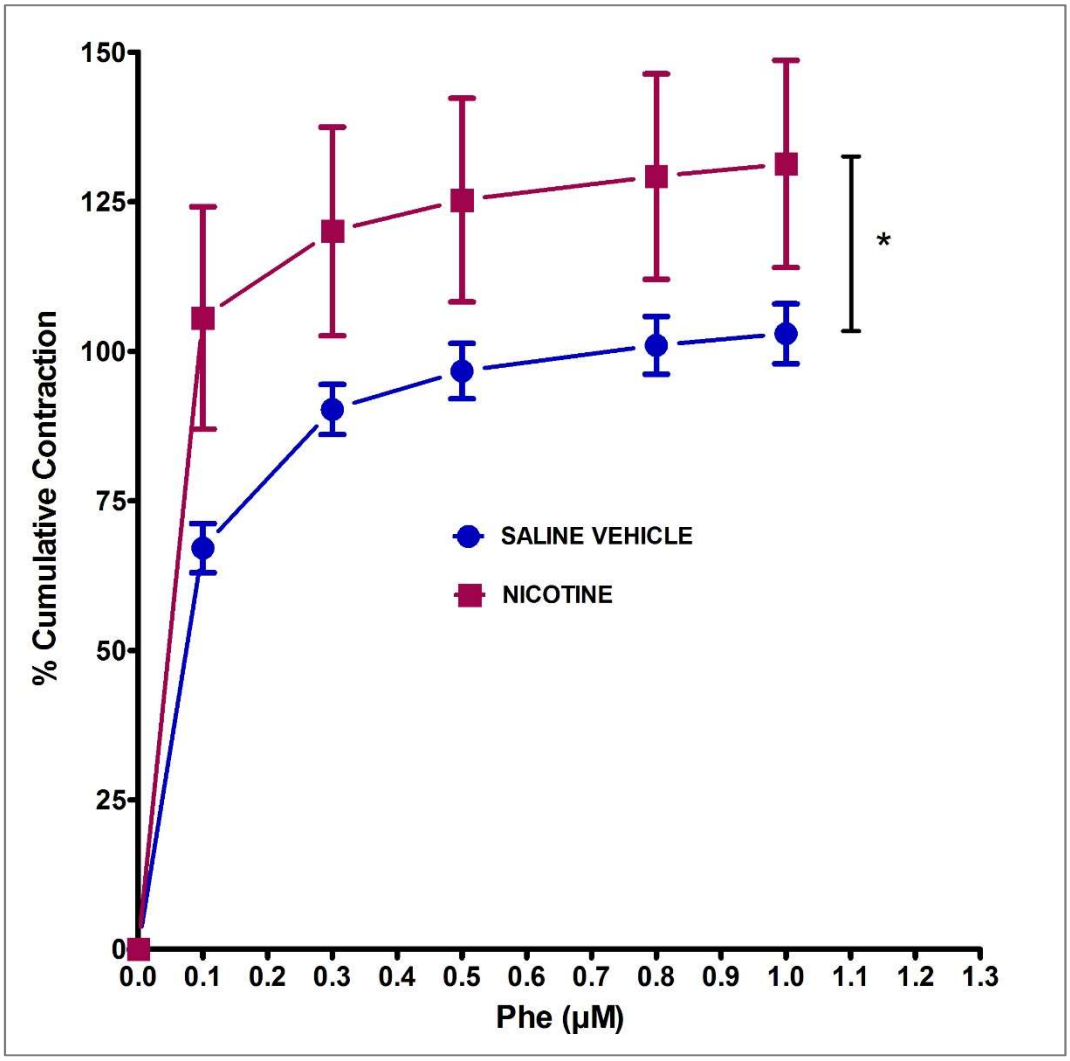

Figure 3.13. Graph indicating the contractile responses of aortic rings harvested from the veh control and nicotine treated rats following cumulative Phe administration (* $p<0.05$ nicotine vs veh control) (n = 6-9 per group).

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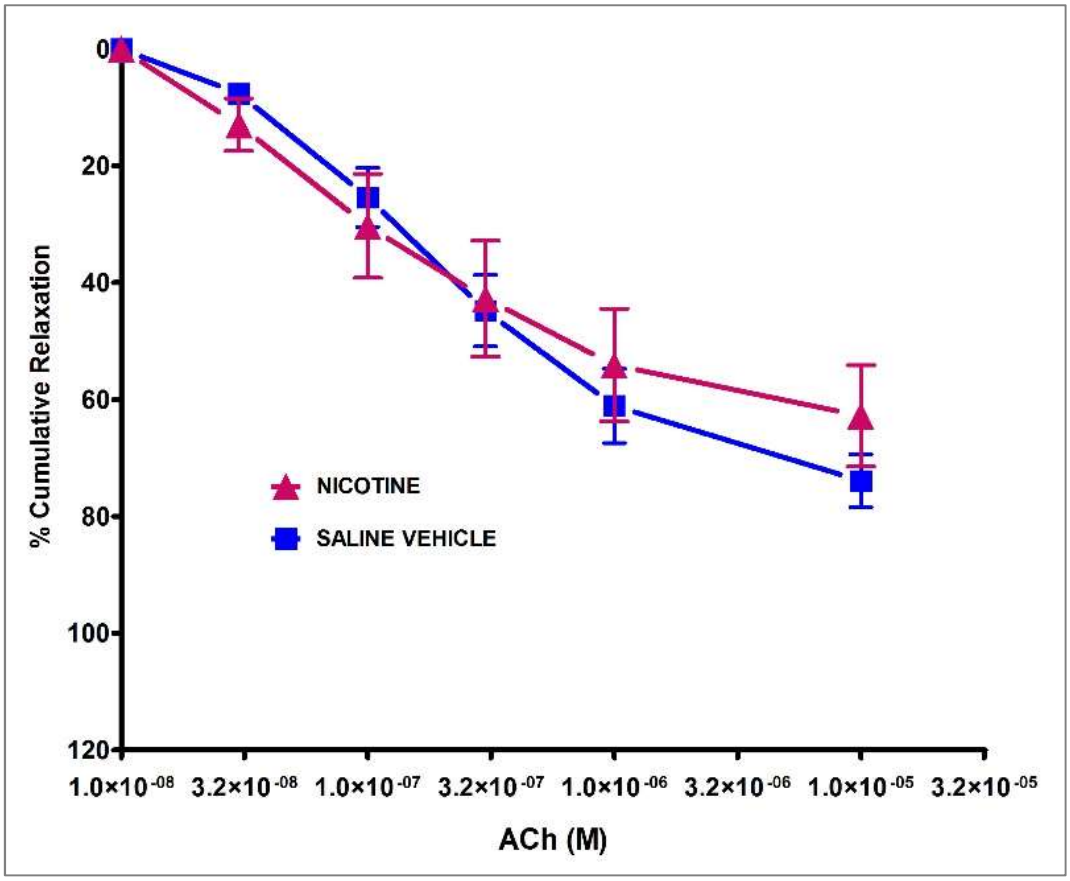

Figure 3.14. Graph indicating the relaxation response of aortic rings harvested from veh control and nicotine treated rats following cumulative ACh administration (n = 6-9 per group).

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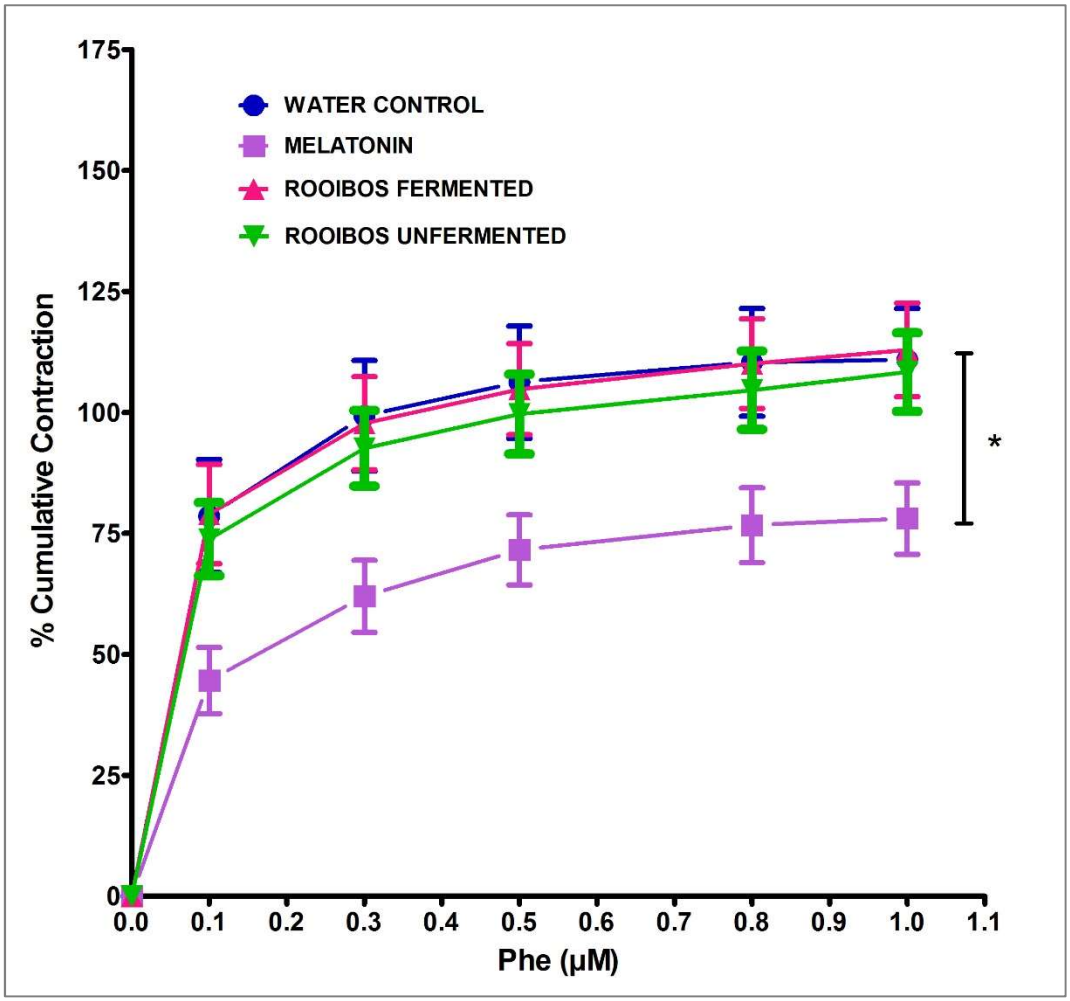

Figure 3.15. Graph indicating the contractile responses of aortic rings from Mel, RF, RUF and water control animals following cumulative Phe administration (* $p<0.05$ Mel vs RF, RUF and water control) (n = 8-10 per group).

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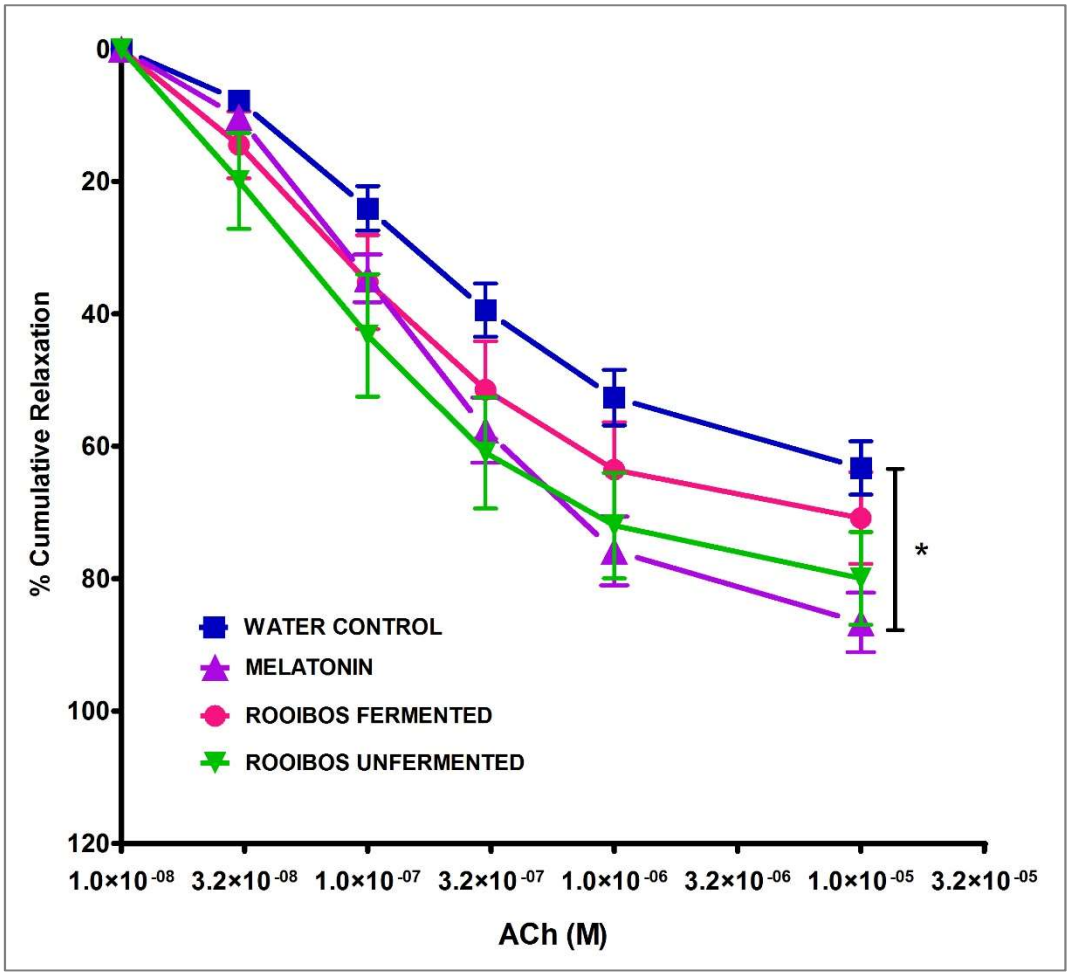

Figure 3.16. Graph indicating the relaxation response of aortic rings harvested from Mel, RF, RUF and water control treated rats following cumulative ACh administration. (* $p < 0.05$ water control vs Mel, RF and RUF) (n = 7-10 per group).

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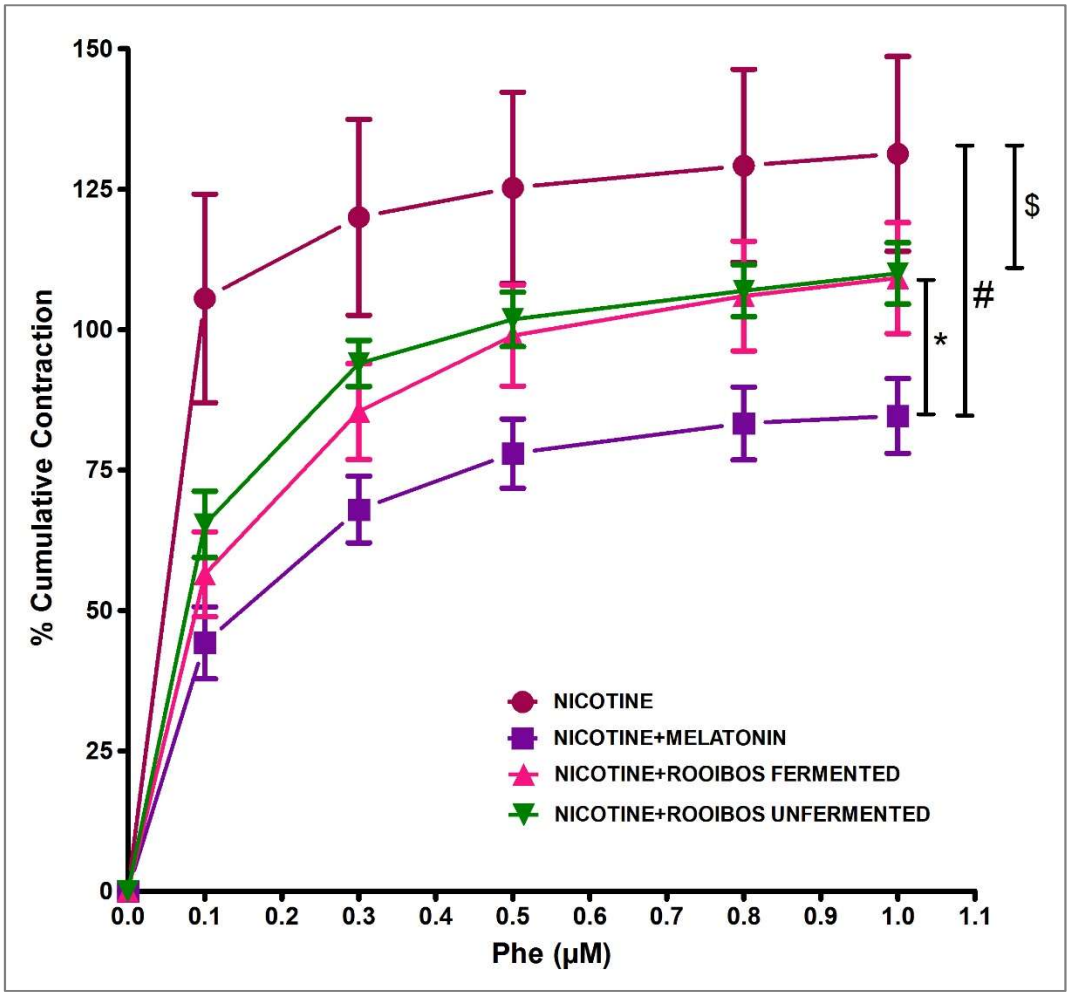

Figure 3.17. Graph indicating the contractile responses of aortic rings harvested from nicotine, NMel, NRF and NRUF treated rats following cumulative Phe administration (* $p<0.05$ NMel vs NRF and NRUF; # $p<0.05$ NMel vs nicotine; $ $p<0.05$ NRF, NRUF vs nicotine) (n = 6-10 per group).

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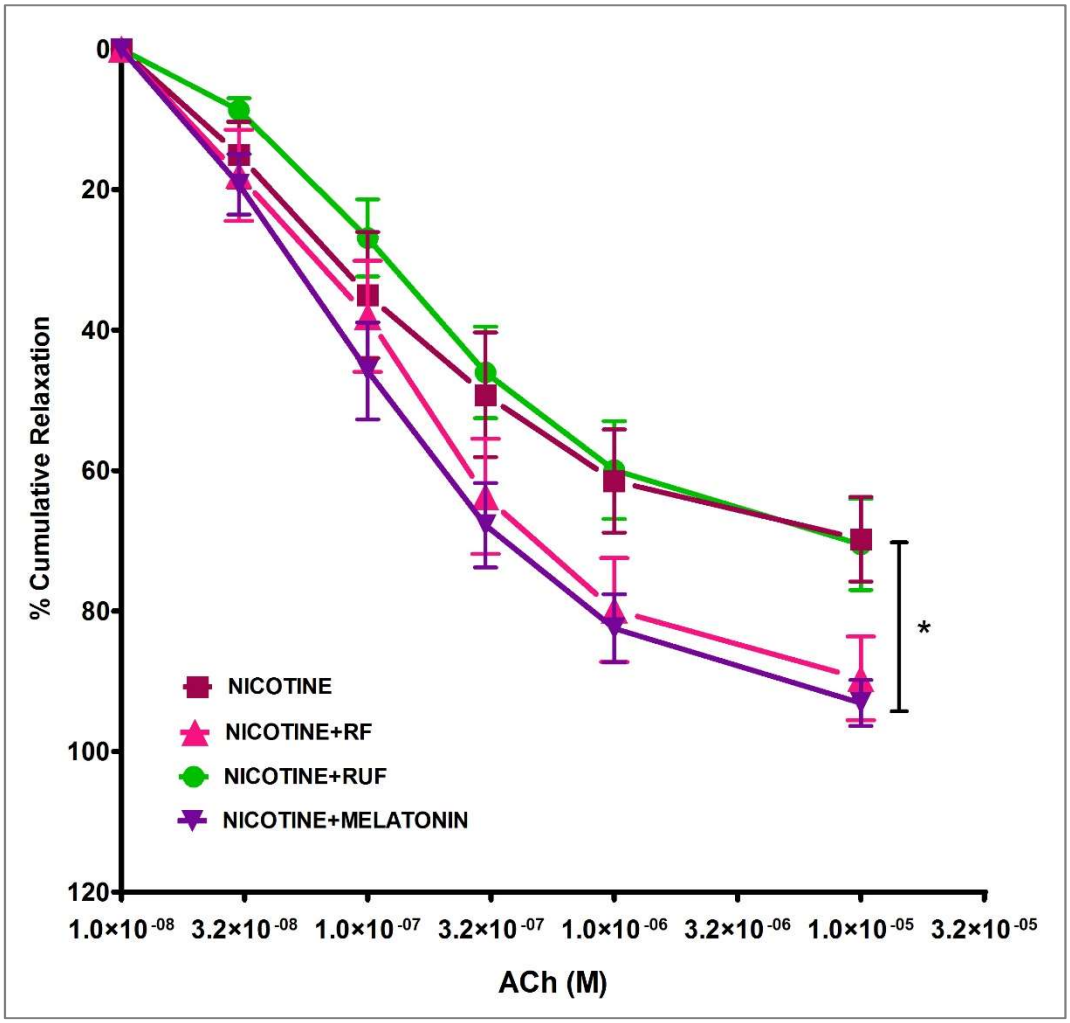

Figure 3.18. Graph indicating the relaxation response of aortic rings harvested from nicotine, NMel, NRF and NRUF treated rats following cumulative ACh administration (* $p<0.05$ NMel and NRF vs nicotine and NRUF) (n = 6-10 per group).

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3.3.3.3.2. Additional in vitro Investigations

3.3.3.3.2.1. The Effects of Nicotine on NO Production and Cell Viability under Baseline Conditions: Dose-response Investigations

In separate *in vitro* investigations carried out on cultured rat aortic endothelial cells (AECs) the effect of 1 µM, 10 µM and 100 µM nicotine on NO production was determined after a 24 hour treatment period. Treatment with 1 µM and 10 µM nicotine had no significant effect on NO production, while 100 µM nicotine showed significantly lower DAF-2/DA fluorescence compared to control (adjusted to 100%) and 1 µM nicotine (96.37 ± 4.24%), indicating a reduction in NO production (79.5 ± 2.98%; $p<0.05$) (Fig 3.19).

In the dose-response investigations, 1 µM and 10 µM had no significant effects on cell viability over a 24 hour treatment period. However, 100 µM nicotine showed a significant reduction in cell viability, as indicated by increased Annexin V fluorescence (162.2 ± 57.16%) (Fig 3.20) and increased PI fluorescence (177.2 ± 30.25%) compared to control (adjusted to 100%) (Fig 3.21).

Based on the fact that 100 µM nicotine treatment induced significant cell injury (reduced NO production and reduced cell viability), this concentration was chosen for further experimentation.

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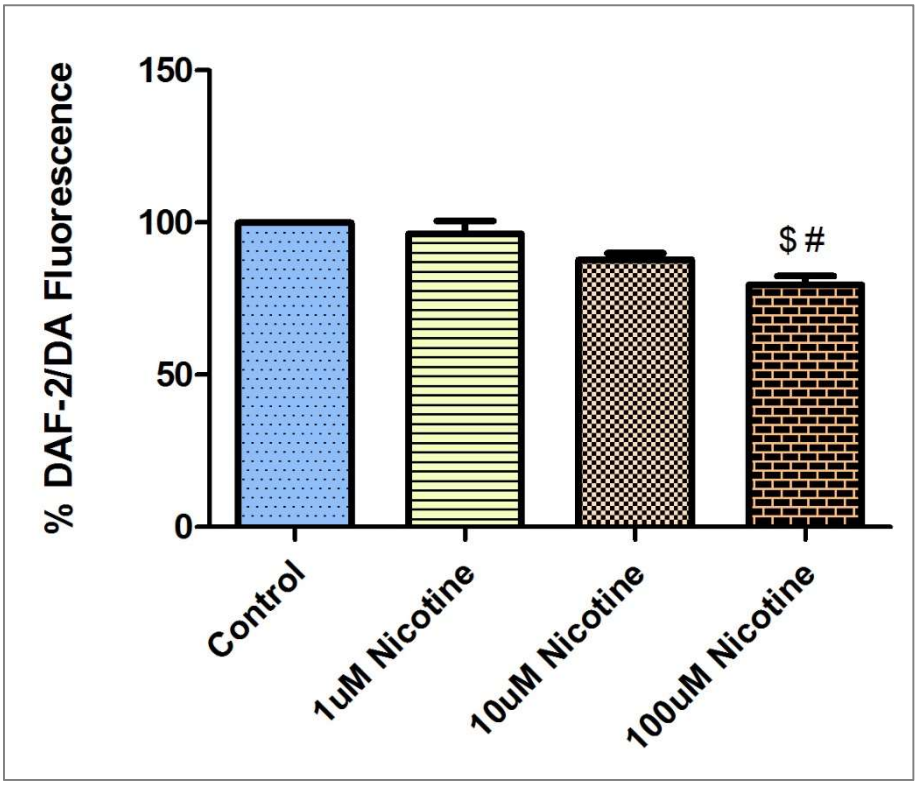

Figure 3.19. The effect of 1 μM, 10 μM and 100 μM nicotine on intracellular NO production as measured by DAF-2/DA fluorescence after a 24 hour treatment period. 100 μM nicotine significantly decreased NO levels after 24 hours. $ $p<0.05$ vs Control; # $p<0.05$ vs 1 μM Nicotine (n = 4-10 per group).

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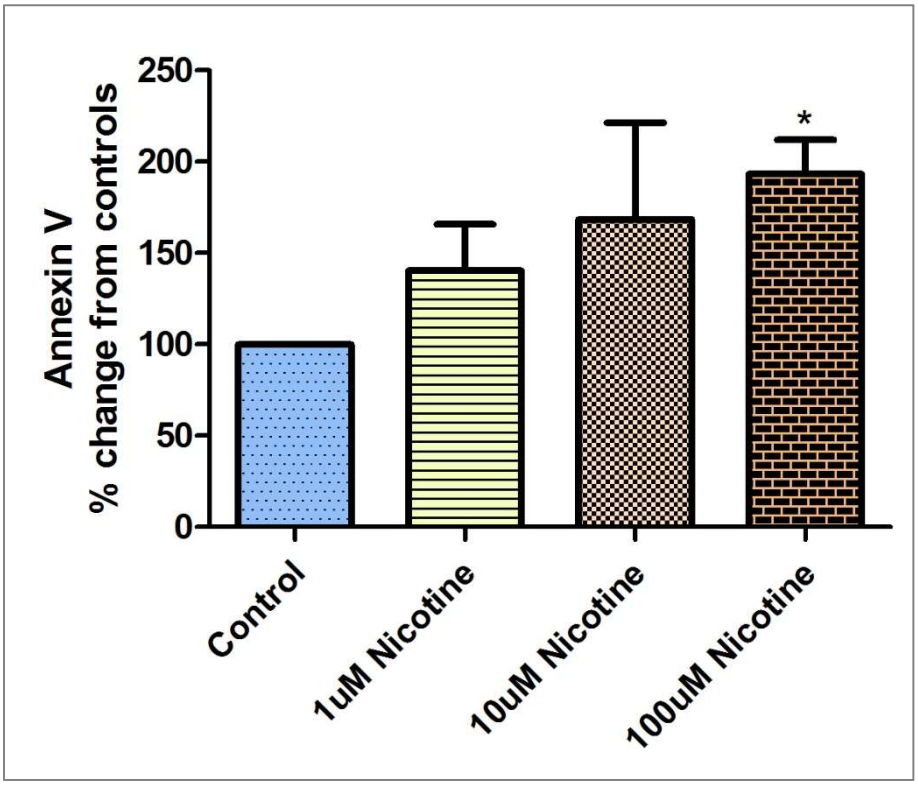

Figure 3.20. The effect of 24 hour nicotine treatment on cell viability. % Change in apoptosis indicated by Annexin V fluorescence. * $p<0.05$ vs Control (n = 4-8 per group).

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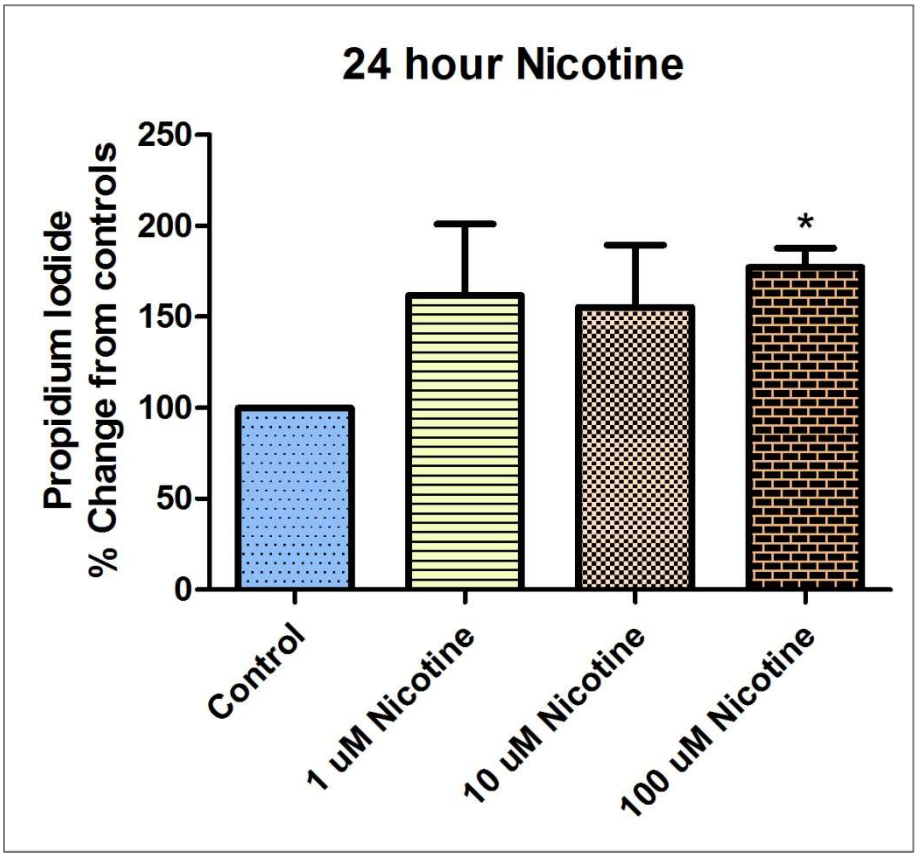

Figure 3.21. The effect of 24 hour nicotine treatment on cell viability. % Change in necrosis indicated by PI fluorescence. * $p<0.05$ vs Control (n = 4-8 per group).

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3.3.3.3.2.2. The Effects of Fermented Rooibos (RF) on NO Production and Cell Viability on Cultured Aortic Endothelial Cells (AECs) under Baseline Conditions: Dose-response Investigations

The effects of 0.015 mg/ml; 0.0625 mg/ml; 0.125 mg/ml and 0.25 mg/ml fermented rooibos on NO production and cell viability (necrosis) were determined under baseline conditions in healthy cells after a 24 hour treatment period.

Fermented rooibos (0.25 mg/ml) resulted in a significant reduction in NO production when compared to the control (81.68 ± 5.80%; $p<0.05$); however, treatment with 0.015 mg/ml, 0.0625 mg/ml and 0.125 mg/ml RF exerted no detrimental effects on NO production compared to control (adjusted to 100%) (0.015 mg/ml: 98.39 ± 5.38%; 0.0625 mg/ml: 96.69 ± 8.06%; 0.125 mg/ml: 90.42 ± 9.44%) (Fig 3.22).

Fermented rooibos (0.125 mg/ml) resulted in a significant increase in necrosis as measured by PI fluorescence when compared to the control (adjusted to 100%) (140.7 ± 6.04%, $p<0.05$). 0.015 mg/ml, 0.0625 mg/ml and 0.25 mg/ml fermented rooibos treatment had no effects on necrosis compared to control, as measured by PI fluorescence (0.015 mg/ml: 91.84 ± 19.63%; 0.0625 mg/ml: 122.2 ± 19.36%; 116 ± 7.71%) (Fig 3.23).

Based on these results, 0.015 mg/ml fermented rooibos was chosen as the concentration for further experimentation, since it did not result in a reduction in NO production and did not affect cell viability.

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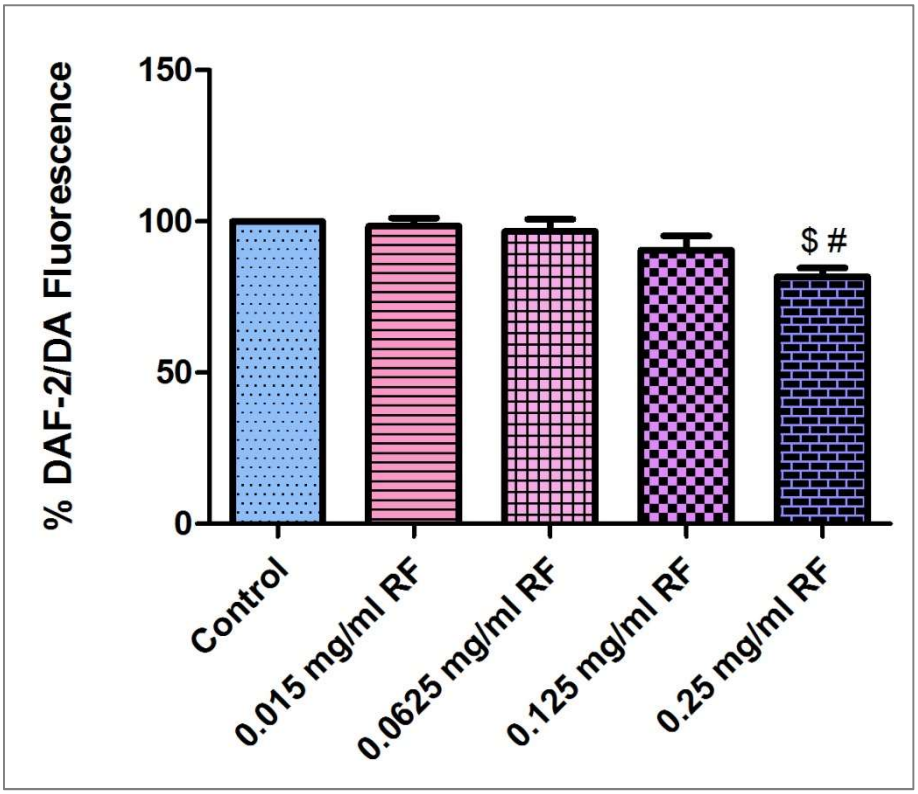

Figure 3.22. The effects of 0.015 mg/ml; 0.0625 mg/ml; 0.125 mg/ml and 0.25 mg/ml fermented rooibos on NO production as measured by DAF-2/DA fluorescence after a 24 hour treatment period. $ $p<0.05$ vs Control; # $p<0.05$ vs 0.015 mg/ml RF (n = 6-8 per group).

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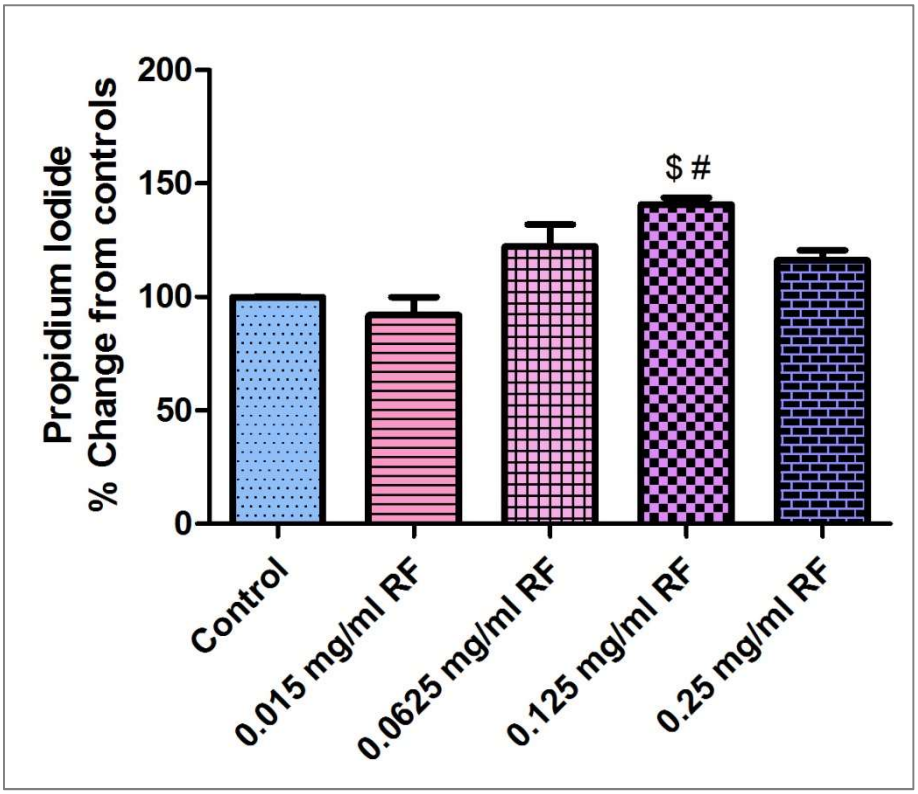

Figure 3.23. The effect of 0.015 mg/ml; 0.0625 mg/ml; 0.125 mg/ml and 0.25 mg/ml fermented rooibos treatment over a 24 hour period on cell viability. % Change in necrosis indicated by PI fluorescence. $ $p<0.05$ vs control; # $p<0.05$ vs 0.015 mg/ml RF (n = 6-8 per group).

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3.3.3.3.2.3. The Effects of RF Pre-treatment on NO Production and Cell Viability in Nicotine Treated AECs

AECs were pre-treated for one hour with 0.015 mg/ml RF, followed by the addition of 100 µM nicotine for a further 24 hours. Pre-treatment with 0.015 mg/ml RF was able to modestly, but significantly increase NO production in nicotine-injured cells compared to cells treated with nicotine only, as indicated by DAF-2/DA fluorescence (control: 100 %; 100 µM nicotine: 78.75 ± 4.65 %; 0.015 mg/ml RF: 98.39 ± 2.69 %; nicotine in combination with RF: 93.5 ± 4.01 %) (Fig 3.24).

However, pre-treatment with 0.015 mg/ml fermented rooibos was not able to significantly reduce necrosis in nicotine-injured cells, as measured by PI fluorescence (Control 100%; 100 µM nicotine: 152.6% ± 32.79%; 0.015 mg/ml rooibos fermented: 91.84% ± 19.63%; nicotine and rooibos fermented co-treatment: 133.8% ± 17.10%) (Fig 3.25).

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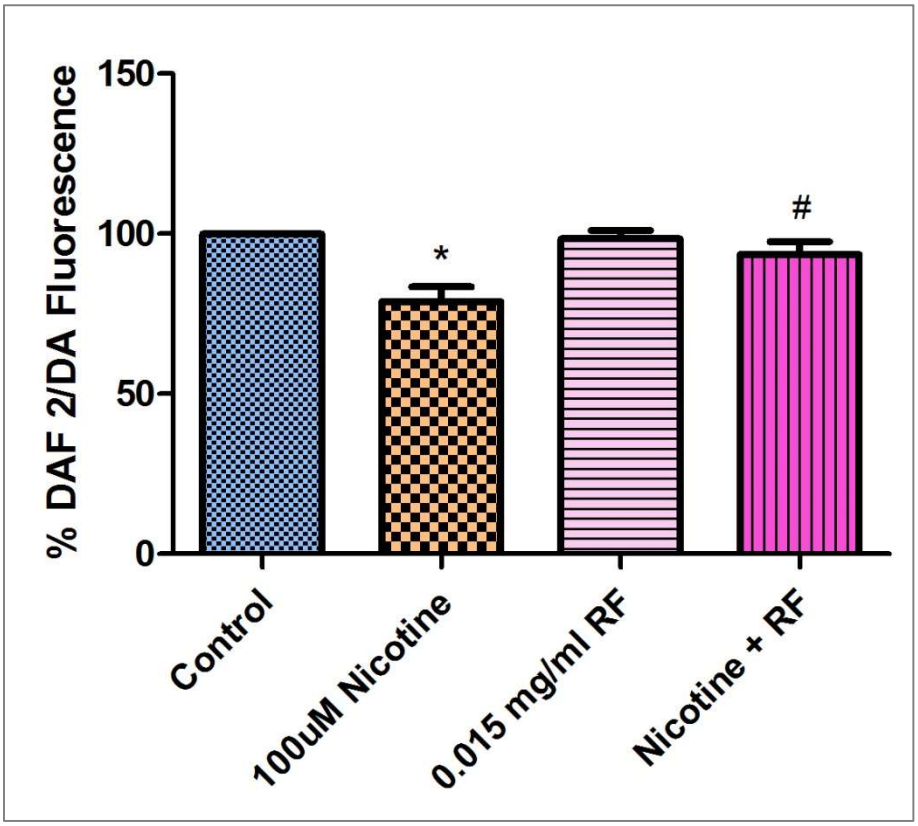

Figure 3.24. The effects of fermented rooibos pre-treatment on NO as measured by DAF-2/DA fluorescence. * $p<0.05$ vs control; # $p<0.05$ vs 100 μM nicotine (n = 6-8 per group).

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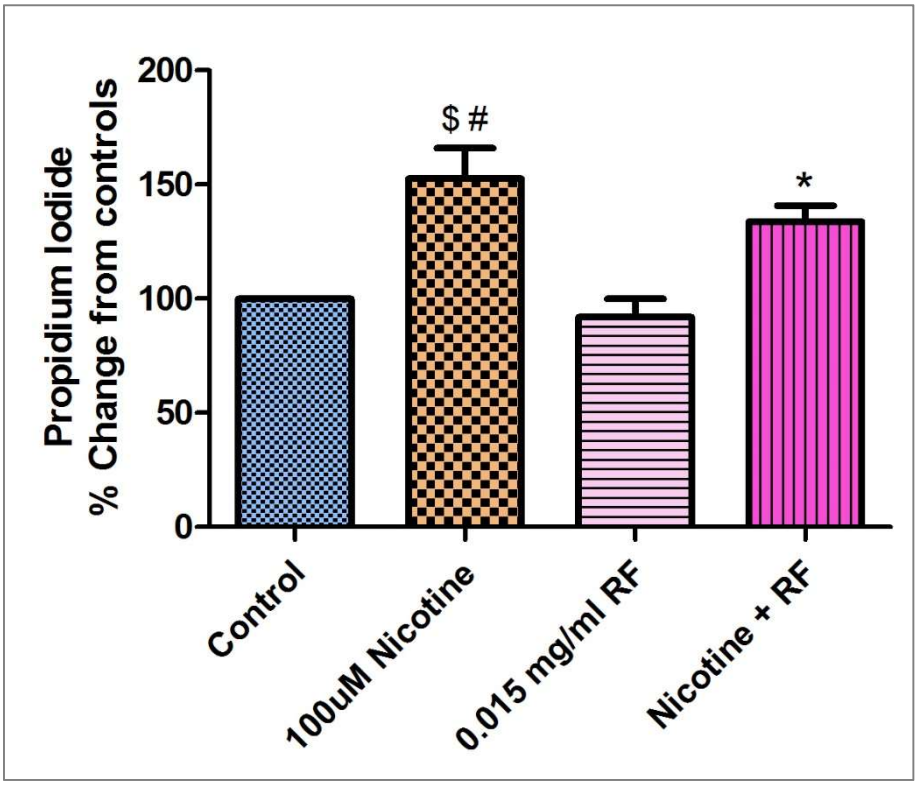

Figure 3.25. The effects of fermented rooibos pre-treatment on cell viability. % Change in necrosis indicated by PI fluorescence. $ $p<0.05$ when compared to control. # $p<0.05$ when compared to 0.015 mg/ml RF. * $p<0.05$ when compared to 0.015 mg/ml RF (n = 6-8 per group).

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3.3.3.4. Antioxidant Enzyme Activity and Oxidative Stress Status Measurements

3.3.3.4.1. Superoxide Dismutase (SOD)

SOD activity was determined in heart tissue homogenates (cytosolic and mitochondrial fractions) and liver tissue homogenates, where one unit (U) of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity was significantly increased in the RF treatment group (73.55 ± 4.89 U/mg protein) of the heart cytosolic fraction, when compared to the water control (57.63 ± 5.26 U/mg protein), RUF (57.57 ± 2.02 U/mg protein), Mel (50.71 ± 4.39 U/mg protein), NRF (49.72 ± 4.19 U/mg protein) and NMel (53.65 ± 2.35 U/mg protein) groups (Fig 3.26). SOD activity was significantly increased in the water control (5.640 ± 0.76 U/mg protein), Mel (5.541 ± 0.63 U/mg protein), NRF (5.445 ± 0.63 U/mg protein) and NRUF (5.766 ± 0.79 U/mg protein) treatment groups of the heart mitochondrial fraction, when compared to the RF group. SOD activity was also significantly increased in the NRUF group, when compared to the veh control group (3.557 ± 0.64 U/mg protein) (Fig 3.27).

SOD activity was significantly increased in the veh control (155.3 ± 6.723 U/mg protein) RF (192.0 ± 21.06 U/mg protein), RUF (182.7 ± 20.38 U/mg protein), NRF (160.7 ± 6.41 U/mg protein) and NMel (180.8 ± 8.96 U/mg protein) treatment groups of liver tissue homogenates, when compared to the nicotine group (121.4 ± 14.69 U/mg protein). SOD activity of the RF group was also significantly increased, when compared to the water control (135.5 ± 5.03 U/mg protein) and Mel (133.8 ± 10.41 U/mg protein) groups (Fig 3.28).

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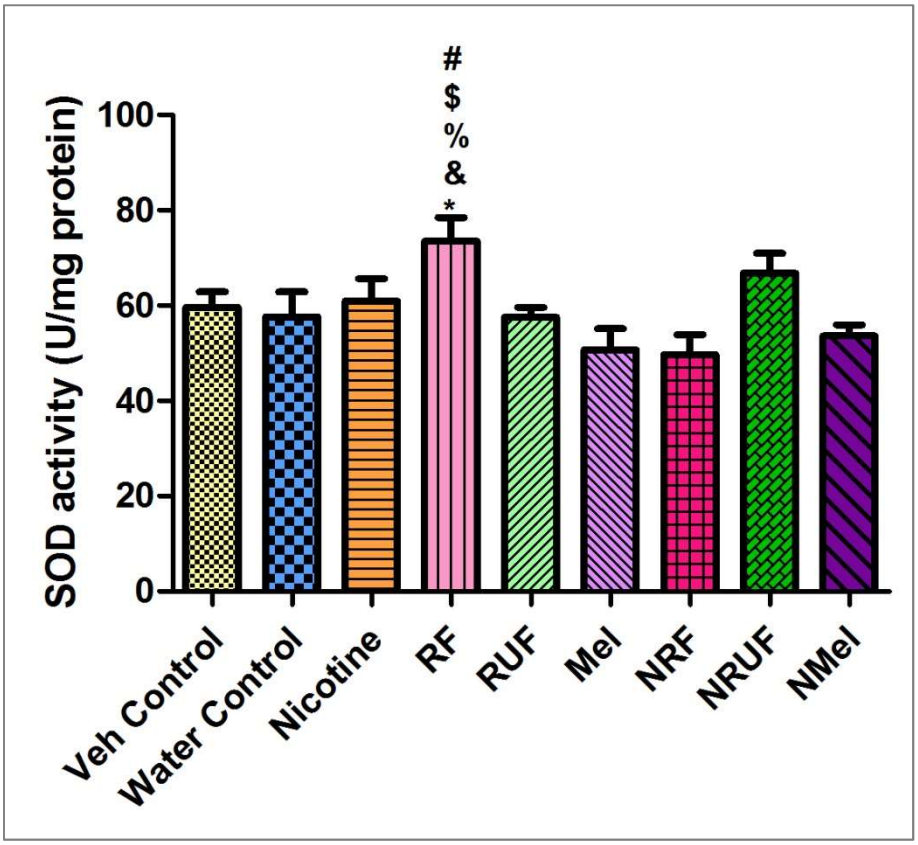

Figure 3.26. SOD activity in heart cytosolic fractions. # $p<0.05$ vs water control; $ $p<0.05$ vs RUF; % $p<0.05$ vs Mel; & $p<0.05$ vs NRF; * $p<0.05$ vs NMel (n = 10 per group).

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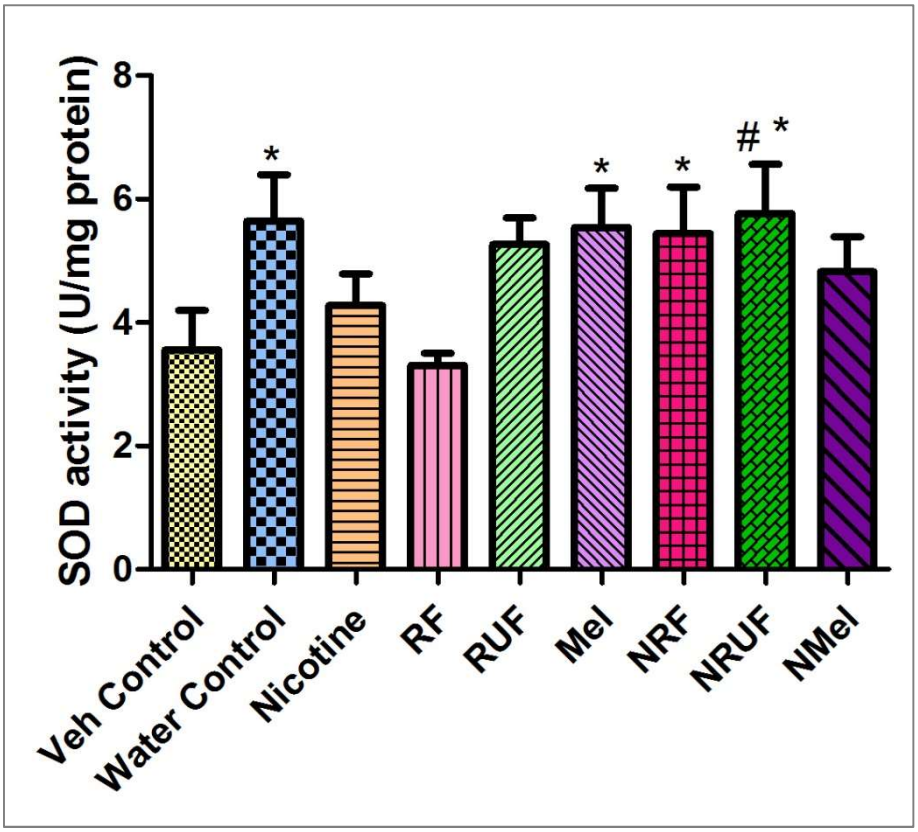

Figure 3.27. SOD activity in the heart mitochondrial fractions. * $p<0.05$ vs RF, # $p<0.05$ vs veh control (n = 10 per group).

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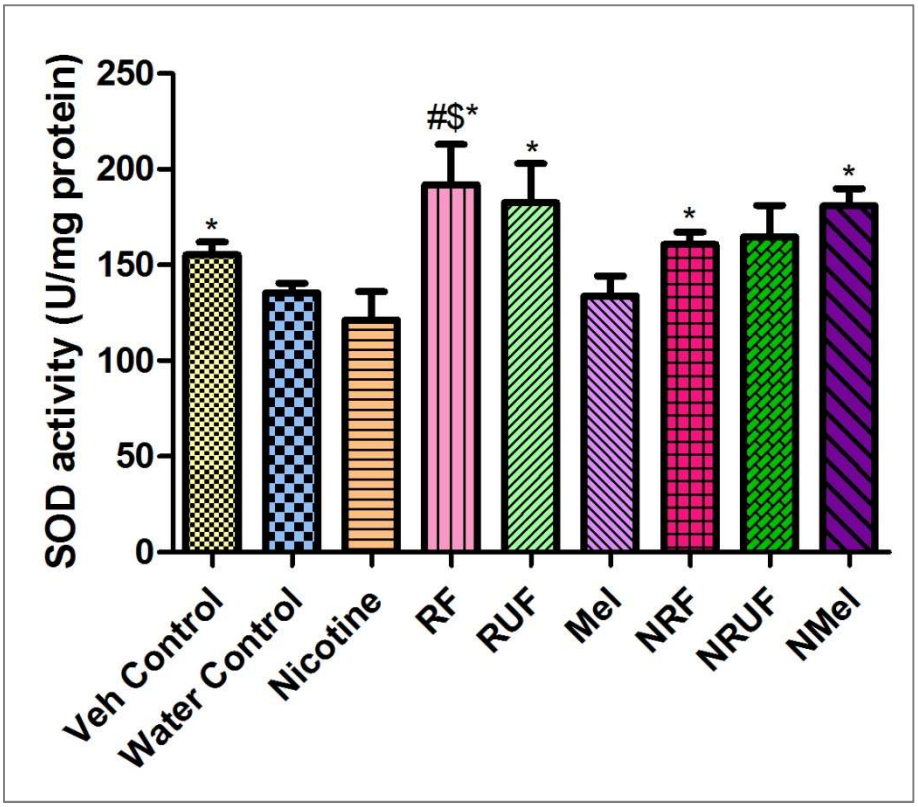

Figure 3.28. SOD activity in liver tissue homogenates; # p<0.05 vs water control, $ p<0.05 vs Mel, * p<0.05 vs nicotine (n = 10 per group).

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3.3.3.4.2. Catalase (CAT)

CAT activity was determined in heart tissue homogenates (cytosolic fraction) and liver tissue homogenates. CAT activity was significantly reduced in the nicotine treatment group (608.2 ± 59.75 µmole/min/µg) of heart tissue homogenates, when compared to the veh control (1109 ± 174.6 µmole/min/µg), RF (878.2 ± 83.69 µmole/min/µg), Mel (997.2 ± 173.6 µmole/min/µg), NRUF (819.1 ± 108.4 µmole/min/µg) and NMel (808.7 ± 71.89 µmole/min/µg) groups (Fig 3.29).

In liver tissue homogenates, CAT activity was significantly reduced in the nicotine treatment group (295.8 ± 76.72 µmole/min/µg) when compared to the vehicle control (868.3 ± 138.4 µmole/min/µg), Mel (597.7 ± 98.19 µmole/min/µg), NRF (727.3 ± 158.6 µmole/min/µg) and NMel (565.8 ± 86.96 µmole/min/µg) groups. CAT activity was also significantly increased in the vehicle control group when compared to the water control group (427.2 ± 51.42 µmole/min/µg) (Fig 3.30).

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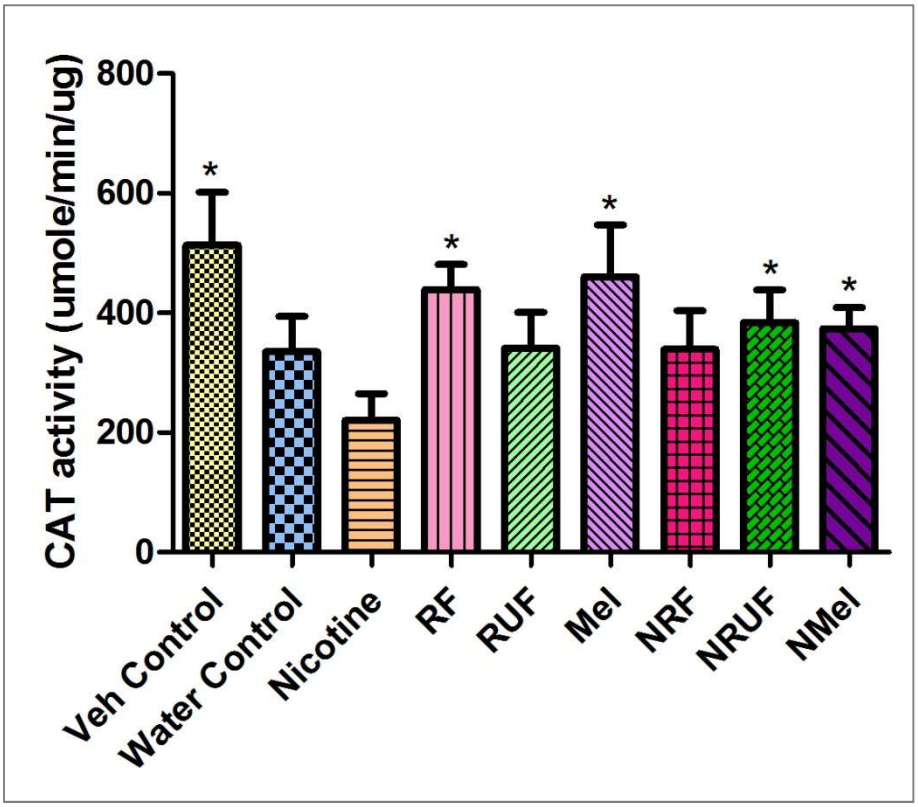

Figure 3.29. CAT activity in the heart cytosolic fractions. * $p<0.05$ vs nicotine (n = 9-10 per group).

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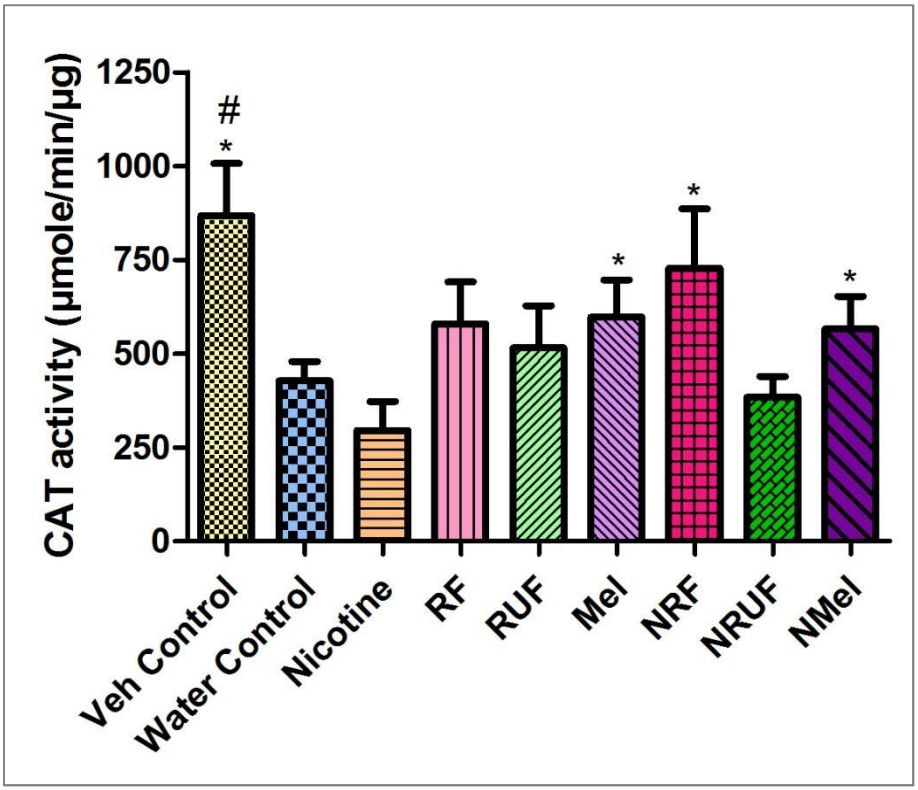

Figure 3.30. CAT activity in liver tissue homogenates. * $p<0.05$ vs nicotine, # $p<0.05$ vs water control (n = 9-10 per group).

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3.3.3.4.3. Glutathione Peroxidase (GPx)

GPx activity was determined in heart tissue homogenates, but did not result in any significant differences between treatment groups (Fig 3.31).

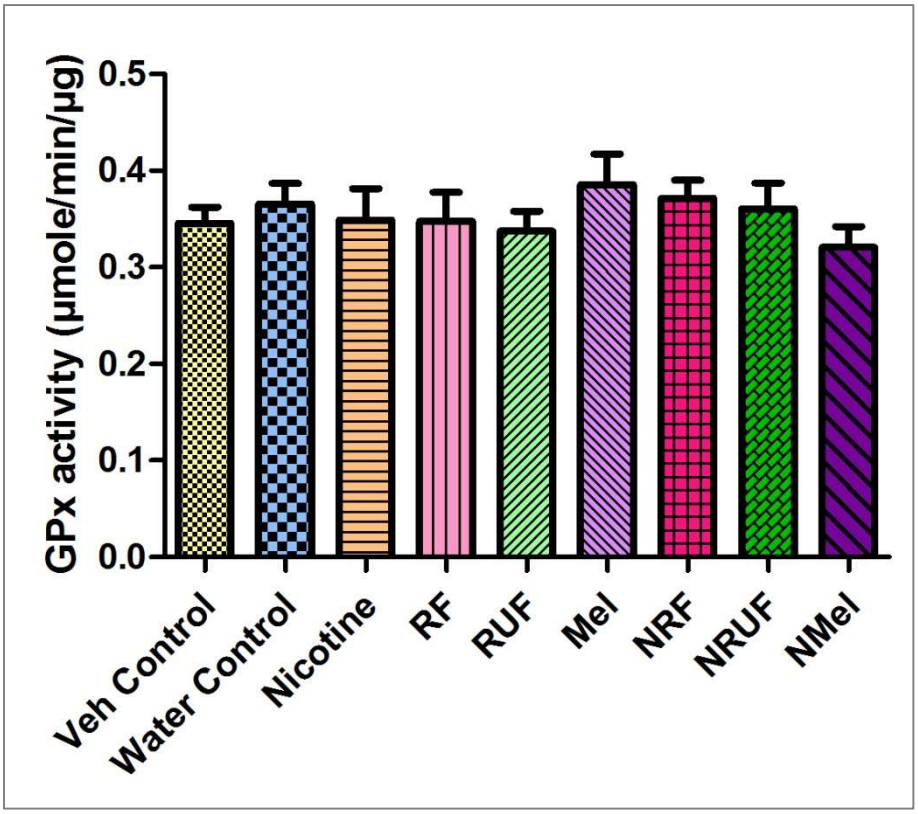

Figure 3.31. GPx activity in heart cytosolic fractions (n = 10 per group).

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3.3.3.4.4. Lipid Peroxidation

Conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) were determined in serum samples as measures of lipid peroxidation. Analysis of CD did not result in any significant difference between treatment groups (Fig 3.32).

TBARS levels were significantly increased in the nicotine treatment group (4.615 ± 0.31 µmol/l) when compared to the vehicle control (2.907 ± 0.23 µmol/l), water control (2.997 ± 0.26 µmol/l), RF (2.472 ± 0.26 µmol/l), RUF (3.350 ± 0.30 µmol/l), Mel (2.829 ± 0.28 µmol/l) and NMel (3.411 ± 0.26 µmol/l) treatment groups (Fig 3.33). TBARS levels were also significantly increased in the NRF (3.772 ± 0.27 µmol/l) and NRUF (3.707 ± 0.14 µmol/l) groups when compared to the RF group.

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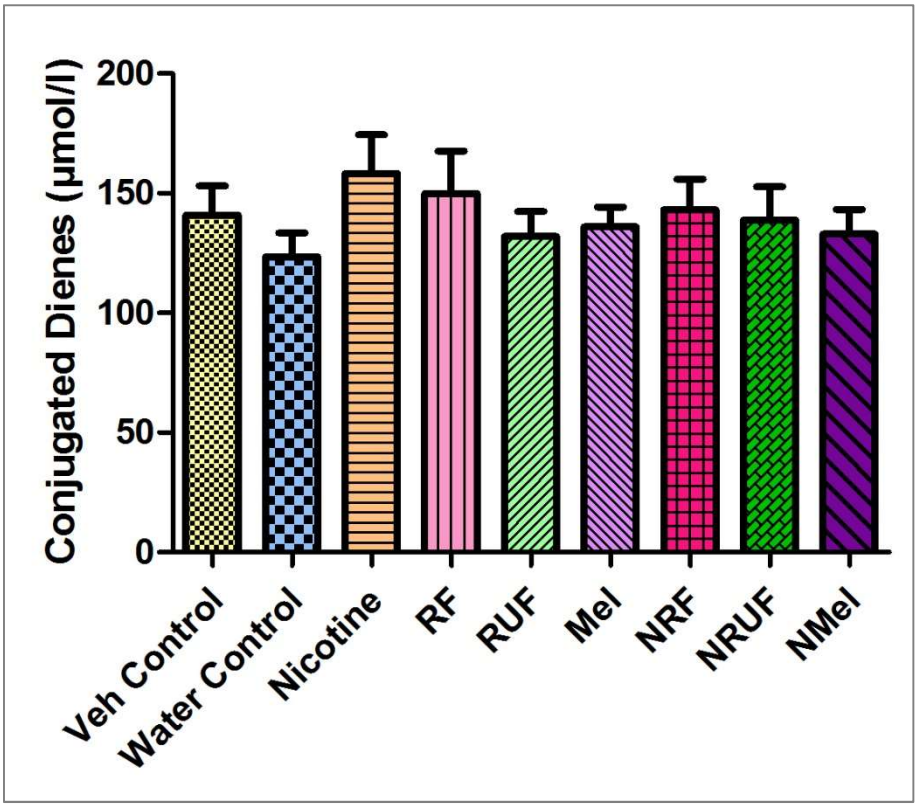

Figure 3.32. Serum CD levels in all treatment groups. No significant differences were observed (n = 9-10 per group).

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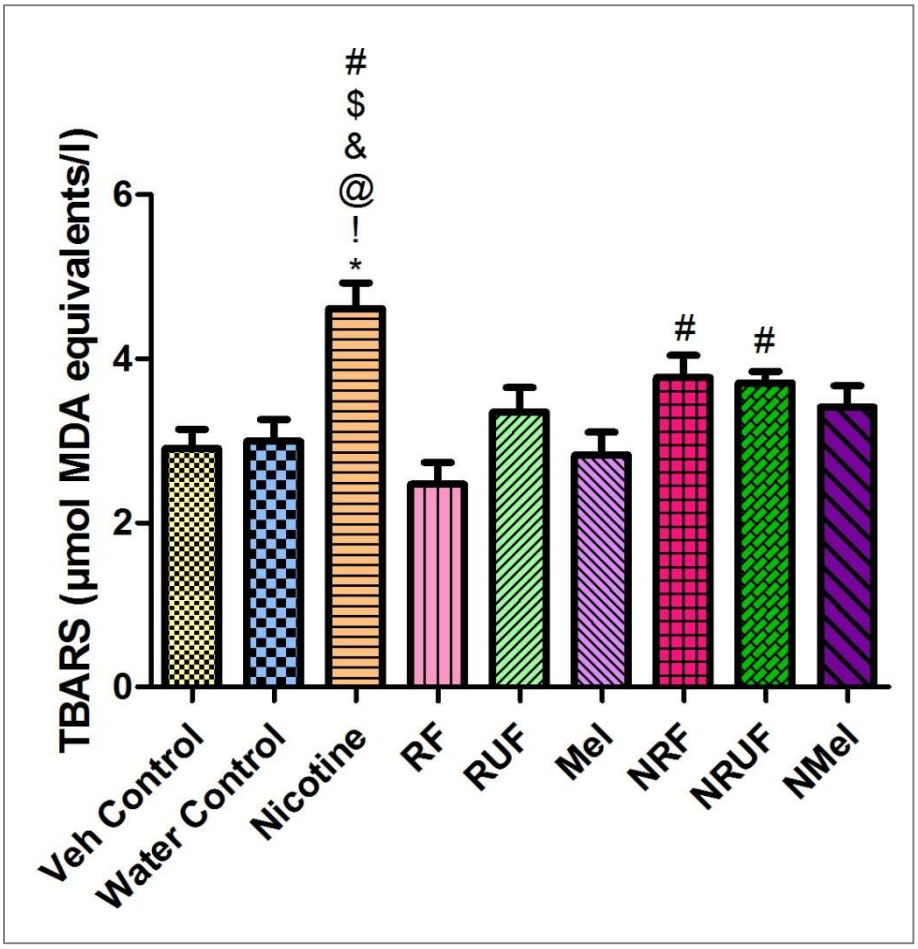

Figure 3.33. TBARS levels in serum of all treatment groups. # p<0.05 vs RF; $ p<0.05 vs veh control; & p<0.05 vs water control; @ p<0.05 vs RUF; ! p<0.05 vs Mel; * p<0.05 vs NMel (n = 10 per group).

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3.3.3.5. Inflammatory Markers

Serum samples were analysed for TNF-α, IL-6 and CRP. TNF-α and IL-6 fell below the detectable range of the multi-analyte kit utilised, however, CRP levels could be determined. CRP levels were significantly decreased in the RUF (293.0 ± 46.8 pg/ml), Mel (257.5 ± 21.0 pg/ml), NRUF (358.3 ± 29.3 pg/ml) and NMel (263.2 ± 58.3 pg/ml) groups, when compared to the veh control (620.4 ± 70.9 pg/ml), water control (774.5 ± 51.5 pg/ml), RF (653.1 ± 29.0 pg/ml) and NRF (680.7 ± 88.5 pg/ml) groups. Additionally, CRP levels in the nicotine treatment group (448.0 ± 69.9 pg/ml) were significantly decreased when compared to the water control group (Fig 3.34).

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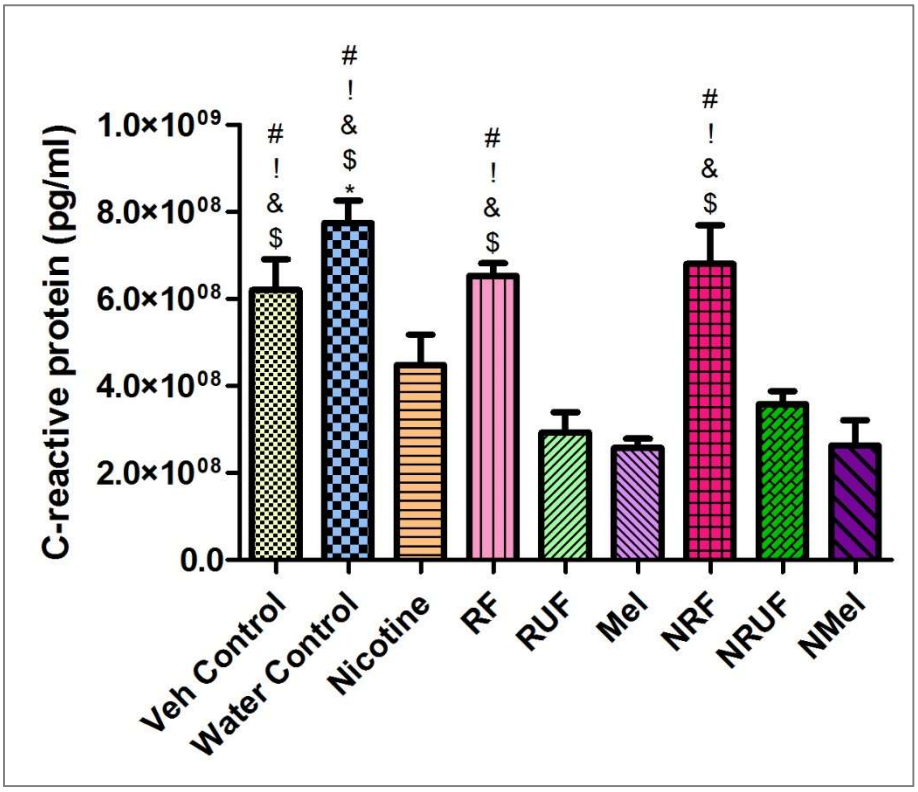

Figure 3.34. C-reactive protein levels in serum. # p<0.05 vs RUF; ! p<0.05 vs Mel; & p<0.05 vs NRUF; $ p<0.05 vs NMel; * p<0.05 vs nicotine (n = 8-10 per group).

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3.3.3.6. Serum Lipids

Total cholesterol (TC), phospholipid (PL) and triglycerides (TG) concentrations were determined in serum. Analysis of TC and PL concentrations did not result in any significant differences (Fig 3.35 and Fig 3.36). TG concentrations were significantly decreased in the nicotine (0.376 ± 0.05 mmol/l) group, when compared to the veh control (0.557 ± 0.07 mmol/l), water control (0.635 ± 0.06 mmol/l), RF (0.639 ± 0.07 mmol/l) and RUF (0.76 ± 0.08 mmol/l) treatment groups. TG concentrations were also significantly decreased in the NMel (0.41 ± 0.05 mmol/l) group, when compared to the RUF group (Fig 3.37).

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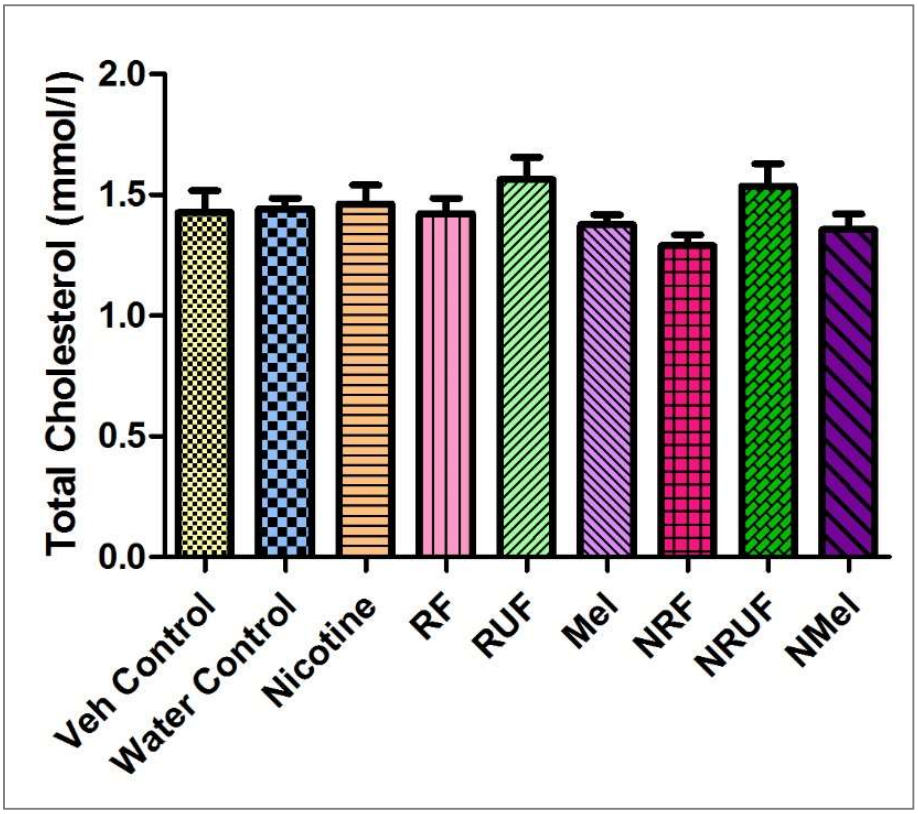

Figure 3.35. Total cholesterol concentrations in serum. No significant differences were observed (n = 10 per group).

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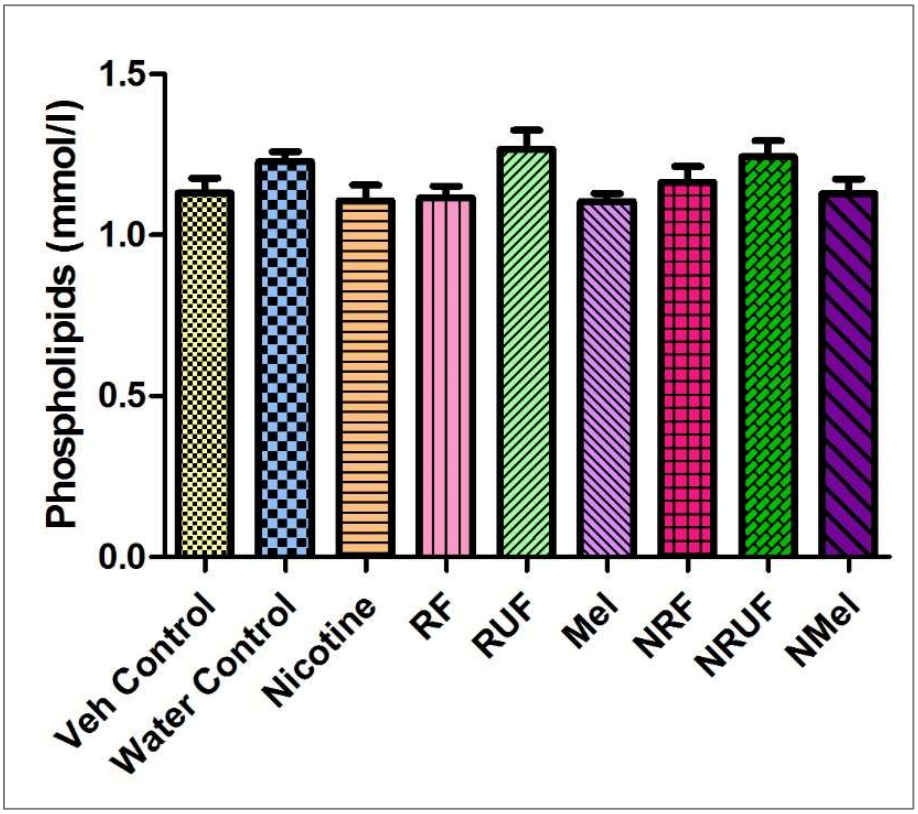

Figure 3.36. Phospholipid concentrations in serum. No significant differences were observed (n = 10 per group).

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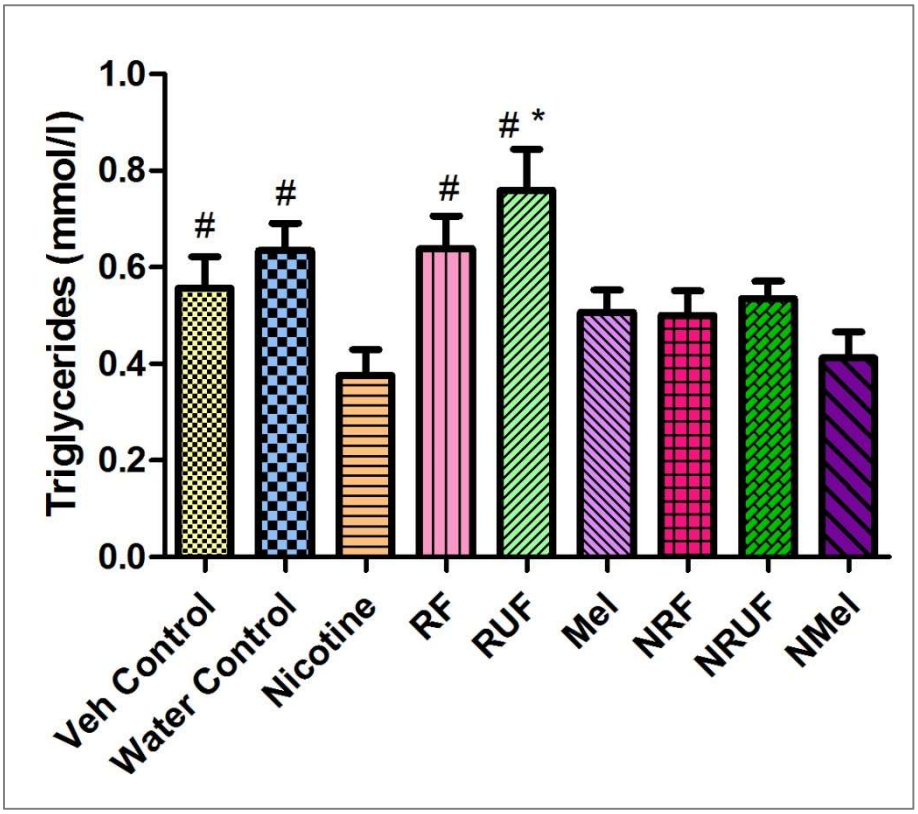

Figure 3.37. Triglyceride concentrations in serum. # $p<0.05$ vs nicotine; * $p<0.05$ vs NMel (n = 10 per group).

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CHAPTER 4: DISCUSSION

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CVD is a leading cause of death globally (Mendis, 2011) and it is expected that the number of annual deaths due to CVD will escalate to 23.4 million by the year 2030 (WHO, 2009). Cigarette smoking is one of the most important risk factors of CVD, while nicotine, the addictive substance in tobacco, is implicated in the pathogenesis of atherosclerosis (Tsiara *et al*., 2003 and Kilaru *et al*., 2001). The relative health of vascular endothelium is a critical determinant of future cardiovascular disease and is involved in many important functions, such as regulation of vascular tone and the synthesis and release of vasoactive substances, thus playing an important role in maintaining vascular homeostasis (Behrendt and Ganz, 2002). NO can be considered one of the most important vasoactive substances and is a potent vasodilator (Mudau *et al*., 2012), in addition to possessing cardioprotective properties. In this regard, two major enzymatic sources of NO, eNOS and nNOS, have previously been shown to have anti-atherosclerotic actions (Liu and Haung, 2008).

Endothelial dysfunction is an early precursor of atherosclerosis and its development is associated with a number of cardiovascular risk factors, including smoking (Cipollone *et al*., 2007). Endothelial dysfunction develops when the balance between the vasodilatory and vasoconstrictory state of blood vessels is disrupted, usually as a result of reduced NO bioavailability. Oxidative stress can lead to a loss of vascular homeostasis and appears to be a major underlying mechanism of endothelial dysfunction. If risk factors are sustained and early endothelial changes are not reversed, progression to atherosclerosis and CVD will follow (Mudau *et al*., 2012). However, it has been shown that early endothelial changes, such as ED, are reversible (Hsueh *et al*., 2004). Thus, it is important to identify therapeutic compounds capable of counteracting oxidative stress, reducing or reversing endothelial dysfunction, and restoring vascular homeostasis.

Nicotine use is associated with the development of CVD and is capable of exerting cardiovascular effects, including an increase in heart rate and myocardial activity (Kilaru *et al*., 2001). Nicotine has been shown to increase blood pressure (Downey *et al*., 1981) and impair flow-mediated vasodilation by decreasing NO bioavailability (Puranik and Celermaier, 2003). Nicotine also affects lipid metabolism by increasing LDL-cholesterol and decreasing HDL-cholesterol, resulting in higher levels of oxidised LDL (OxLDL) (Clutte-Brown *et al*., 1986, Harats *et al*., 1989).

In view of the above, this dissertation set out to explore the putative vascular, endothelial and metabolic protective effects of two compounds, viz. rooibos and melatonin in male Wistar rats exposed to nicotine administration. Both the indigenous South African shrub, rooibos, and the hormone melatonin (which is also pharmaceutically synthesised) are known antioxidants and possess properties that could modulate nicotine-induced vascular injury. Rooibos is a popular

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herbal beverage, of which both fermented and unfermented forms exist. Rooibos possesses biological properties (Robak and Gryglewski, 1996) and exerts potent antioxidant, immune-modulating and chemo-protective actions (McKay and Blumberg, 2007). Both fermented and unfermented rooibos are able to increase plasma antioxidant systems (Villano *et al*., 2010), while it has been shown that consumption of fermented rooibos can improve the lipid profile and redox status (Marnewick *et al*., 2011). Cardioprotective effects of rooibos have previously been demonstrated in both human and animal studies (Marnewick et al., 2011, Pantsi *et al*., 2011), as well as in *in vitro* models (Dludla *et al*., 2014), however, the effects of rooibos on endothelial and vascular health are lacking. Melatonin, the hormone responsible for controlling circadian rhythms, is secreted in a variety of organs and tissues in addition to the pineal gland (Claustrat *et al*., 2005, Slominski *et al*., 2012). Melatonin is involved in many physiological processes in humans and animals, including blood pressure control, regulation of the immune response and free radical scavenging (Hardeland *et al*., 2006, Rodella *et al*., 2013). The chronic administration of melatonin under experimental conditions was demonstrated to be cardioprotective, which can be attributed to its free radical scavenging and antioxidant properties (Lochner *et al*., 2006, Nduhirabandi *et al*., 2011).

This dissertation made use of an *in vivo* model of male Wistar rats and an *ex vivo* model of rat aortic ring segments, complemented by an *in vitro* model of cultured aortic endothelial cells. Biometric data and fluid intakes, blood pressure measurements, vascular function supported by *in vitro* investigations, as well as antioxidant enzyme activity and oxidative stress status, inflammatory cytokines and serum lipids were determined as measures of the effects of nicotine in the rat model. The effects of nicotine co-treated with rooibos (fermented and unfermented) or melatonin were assessed according to the same endpoints to determine whether the observed nicotine-induced effects could be ameliorated.

4.1. Phytochemical Content of Rooibos

Analysis of 2% RF and 2% RUF used in our study revealed a significantly higher soluble solid, total polyphenol and flavanol content in RUF, however, RF had a significantly higher flavonol content (Table 3.1, Table 3.3). The main difference between RUF and RF was in the aspalathin and nothofagin contents, which was considerably higher in RUF (Table 3.5). This is consistent with previous findings, since it has been shown that the amount of aspalathin can decrease by almost 98% during fermentation (Schulz *et al*., 2003). Another notable difference in the composition of rooibos used in the present study is the presence of the antioxidant and free radical scavenger, ferulic acid in RF, but not in RUF.

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Studies into the bioavailability of rooibos flavonoids are scarce, however, certain compounds, including aspalathin and nothofagin have been analysed in humans. It was shown that flavonoids from both fermented and unfermented rooibos have a low bioavailability (Stalmach *et al*., 2009, Courts and Williamson, 2009). In another human study, unfermented rooibos and an isolated active fraction of unfermented rooibos were administered to healthy male volunteers and urine and blood samples collected and analysed. Intact aspalathin and nothofagin, as well as metabolites of aspalathin and nothofagin were identified in urine samples, while the main metabolite excreted was methylated aspalathin. In plasma, unchanged rooibos flavonoids (including aspalathin) from unfermented rooibos were detected in trace quantities (Breiter *et al*., 2011). It has also been shown that most aspalathin metabolites are excreted within five hours of rooibos consumption in humans, which suggests absorption in the small intestine. It has been suggested that most rooibos flavonoids pass through from the small intestine to the large intestine, where it comes into contact with colonic microflora, which cleave flavonoids to produce low molecular weight phenolic acids (Stalmach *et al*., 2009). Furthermore, it has been suggested that these catabolites are absorbed into the portal vein, pass through the body and then excreted (Roowi *et al*., 2009).

4.2. *In vivo* and *ex vivo* Investigations: Pilot Study

Prior to investigating the effects of rooibos and melatonin, a pilot study was undertaken to determine the optimal injury-inducing nicotine concentration in our experimental setting. According to Benowitz and Jacob (1999), the daily nicotine intake of habitual smokers is in the range of 1 mg/kg bw/day nicotine. A dose as low as 0.6 mg/kg bw/day nicotine, which is equivalent to that of a light smoker, has been used to demonstrate that chronic nicotine administration could impair aortic reactivity in Sprague-Dawley rats (Zainalabidin *et al*., 2014). A dose of 2 mg/kg bw/day nicotine was utilised in Wistar rats over a four week treatment period to induce vascular endothelial dysfunction. Treatment with benfotiamine was able to attenuate nicotine-induced vascular endothelial dysfunction, where reduction of oxidative stress and enhanced generation of NO was suggested as mechanisms of action (Balakumar *et al*., 2008b). Therefore, we utilised dosages of 1 mg/kg bw/day nicotine and 2 mg/kg bw/day nicotine in our pilot study.

Endpoints examined included biometric measurements and fluid intakes, blood pressure, vascular function, as well as lipid peroxidation. At the end of the six week treatment period, fluid intake was the only endpoint to be significantly affected. Both 1 mg/kg bw/day and 2 mg/kg bw/day nicotine resulted in a significant reduction in mean daily fluid intake when compared to the saline vehicle control (Fig 3.2), which agrees with previous findings of studies

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that showed a reduction in water intake in nicotine-exposed rats (Clarke and Kumar, 1984). From the pilot study findings, is seems that relatively low nicotine concentrations, were not sufficient to induce injury in the majority of the endpoints in young, healthy male rats over the treatment period.

Based on these results, it was decided to perform the main study with a higher nicotine concentration, namely 5 mg/kg bw/day. A nicotine dose of 5 mg/kg bw/day has previously been used over a treatment period of five weeks and demonstrated that the rate of endothelial cell loss was greater in the nicotine-affected endothelium compared to untreated control (Zimmerman and McGeachie, 1985).

4.3. *In vivo* and *ex vivo* Investigations: Main Study

4.3.1. Biometric Measurements and Fluid Intake

At the end of the six week treatment period, all groups receiving nicotine (nicotine, NRF, NRUF and NMel) had a significantly lower mean body weight when compared to the veh control, water control, RF and RUF groups (Fig 3.6). In addition, the mean body weight gain in the water control, RF and RUF treatment groups were also significantly increased when compared to the veh control and Mel groups. The lower mean body weight gain of nicotine and nicotine co-treatment groups are consistent with previous observations (Kramer *et al*., 2007, Audi *et al*., 2006, Rezvanipour *et al*., 2011). A lower mean body weight gain in nicotine treated rats is often linked to a reduction in food consumption (Ijomone *et al*., 2014). However, food intake, which is a helpful parameter in explaining body weight, was not measured in the current study. In an experimental model of chronic nicotine administration in rats, it was shown that a reduction in body weight can, in part, be linked to nicotine's actions on catecholamenergic neurons within or extending through the perifornical hypothalamus (PFH) (Kramer *et al*., 2007). These findings were supported by studies demonstrating that the administration of a nicotine receptor antagonist, mecamylamine (MEC), could prevent reduced food intake and reduced body weight associated with nicotine administration (Guan *et al*., 2004).

In a previous study chronic melatonin administration in rats lead to reduced body weight gain, without having an effect on metabolism. In the same study, melatonin did not influence food or fluid intake (Terrón *et al*., 2013). Chronic melatonin consumption has also been shown to reduce body weight gain and visceral adiposity in a rat model of prediabetic diet-induced obesity (Nduhirabandi *et al*., 2011). Nicotine administration has also been shown to affect body composition by reducing visceral adiposity. Therefore, a reduction in visceral adiposity could account for reduced body weight gain in the nicotine, as well as melatonin treatment groups. Rooibos has been shown to have anti-obesity potential in hyperlipidaemic mice,

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where the activation of AMP-activated protein kinase (AMPK) and resulting regulation of cellular energy homeostasis was suggested to be a possible mechanism for the amelioration of metabolic disturbances (Beltrán-Debón *et al*., 2011). The anti-obesity potential of rooibos is supported by an *in vitro* model of adipocyte differentiation, in which fermented rooibos was able to inhibit adipogenesis and affect adipocyte metabolism (Sanderson *et al*., 2014). It is therefore not surprising that melatonin and rooibos co-treatment was unable to counter the effects of nicotine in the NMel, NRF and NRUF groups. Furthermore, the reduced mean weight gain observed in the Mel group, when compared to the RF and RUF could indicate that melatonin has a greater potential for reducing body weight gain.

In view of nicotine's known cardiac and hepatic effects, it was decided to investigate whether nicotine administration exerted any macroscopic changes, as determined by changes in the heart and liver weights of the rats. Studies examining the heart weight/body weight and liver weight/body weight ratios are lacking in the context of nicotine co-treated with either rooibos or melatonin. Overall, differences in mean heart weight/body weight and mean liver weight/body weight were modest and nicotine treatment had no significant effect when compared to its vehicle control, indicating that the differences observed might not be of physiological relevance (Fig 3.7 and Fig 3.8). Cigarette smoke exposure has previously been shown to increase heart weights in male and female rats in 13-week smoke exposure studies (Gaworski *et al*., 1998, Heck *et al*., 2002), while a moderate increase in heart weight/body weight ratio has been observed in mice, which was attributed to the potential anorectic effects of nicotine (Colombo *et al*., 2013). Nicotine is metabolized extensively by the liver and nicotine treatment for 60 days has previously been shown to increase liver weights in rats, which was attributed to cellular damage leading to the accumulation of lipids in hepatic cells over long-term nicotine exposure (Iranloye and Bolarinwa, 2009). The mean daily fluid intake of the nicotine treatment group was significantly reduced compared to the veh control group (Fig 3.9), which is consistent with previous findings where subcutaneous nicotine administration was able to depress water intake in rats, where water retention was suggested to supress fluid intake (Clarke and Kumar, 1984). The reduced mean daily fluid intake observed in the nicotine treated animals was not affected by co-treatment with RF or RUF; however, melatonin co-treatment was able to restore nicotine's effects on fluid intake to veh control levels. While this is an interesting finding, it does not contribute to the understanding of the modulating properties of melatonin in the context of this study. Melatonin has previously been shown to temporarily increase water intake in male Wistar: Han rats treated with 4 µg/ml of melatonin, when compared to tap water controls (Kassayová *et al*., 2006). The reduced fluid intake of the water control group is an unexpected finding. The water control group did not present with reduced mean weight gain and had the second highest mean weight gain at the end of the six

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week treatment period. The gain in body weight could be attributed to higher food consumption than groups with a lower mean weight gain at the end of the treatment period. Rats had free access to standard rat chow, which has a moisture content of approximately 12% (Allers *et al*., 2008). Therefore, when more food is consumed, moisture is also taken in, which could explain why rats in the water control group had a lower mean fluid intake. Once again, monitoring food consumption would have been a valuable tool in explaining findings.

A reduction in body weight associated with nicotine administration has been directly linked to supressed food intake (Bellinger *et al*., 2003). The ability of nicotine to reduce food and fluid intake is not completely understood, but it has been demonstrated that nicotine reduces food intake by acting on nicotine receptors located in brainstem neurons, indicating that nicotine may act to increase satiety (Guan *et al*., 2004). In a study which aimed to examine the effect of subcutaneous nicotine administration on food and water intake, both food and water intake were supressed. It was suggested that nicotine can supress fluid intake by promoting water retention. Rats were also observed to refrain from drinking water for approximately two hours after subcutaneous injection with nicotine (Clarke and Kumar, 1984).

4.3.2. Blood Pressure

In the present study, blood pressure was non-invasively measured by means of the CODA™ tail-cuff system. The accuracy of the CODA™ VPR non-invasive blood pressure monitoring system has been verified for blood pressure measurements in rodents over the physiological range of blood pressure (Feng *et al*., 2008). Blood pressure measurements using the CODA™ system do not require animals to be sedated or fitted with surgical implants. Our data show that blood pressure was significantly increased in the nicotine treatment group when compared to the veh control group (Fig 3.10, Fig 3.11, Fig 3.12) and that melatonin co-treatment significantly decreased mean diastolic pressure and mean arterial pressure compared to the nicotine treatment group. Unexpectedly, mean systolic pressure was significantly increased in the water control and RUF treatment groups when compared to the veh control group.

Chronic nicotine exposure has been shown to lead to hypertension through various mechanisms (Balakumar and Kaur, 2009). Nicotine can elevate blood pressure indirectly by acting as a sympathomimetic agent by releasing catecholamine locally from adrenergic axon terminals (Mayhan and Sharpe, 1999, Benowitz, 2001). Nicotine has also been shown to play a modulating role in the renin angiotensin aldosterone system (RAAS), whereby nicotine decreased angiotensin converting enzyme 2 (ACE 2) levels in an *in vivo* model (Ferrari *et al*., 2007). ACE 2 is responsible for converting angiotensin-II (Ang-II) to angiotensin 1-7 (Ang 1-7). Ang 1-7 have vasodilatory and anti-proliferative effects, which are important in the control

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of blood pressure (Balakumar *et al*., 2008a). In addition to preventing the favourable conversion of Ang-II to Ang 1-7, nicotine is also capable of increasing plasma levels of the vasoconstrictor peptide, arginine vasopressin (Yu *et al*., 2008), as well as upregulating endothelin-1 (ET-1), which is involved in vasoconstriction and vascular remodelling (Letizia *et al*., 1997, Takashi *et al*., 2004). The increase in ROS associated with nicotine results in a decrease in the bioavailability of NO (Toda and Toda, 2010), leading to an upregulation of Ca^{2+} channels and downregulation of Ca^{2+}-active K^{+} (BK) channels, thereby downregulating NO-induced vasorelaxation (Gerzanich *et al*., 2001). As such, blood pressure is an important and multi-faceted indicator of vascular health in nicotine exposure. As observed in the present study, chronic exposure to nicotine has been shown to significantly increase systolic blood pressure in rats, as measured by the tail cuff method (Moon *et al*., 2013). A significant increase in blood pressure was also reported in a nicotine treatment group in a Wistar rat model of hypercholesterolemia (Tamaoki *et al*., 2009).

Melatonin has previously been shown to play an important role in controlling blood pressure (Rodella *et al*., 2013). Vascular relaxation after acute melatonin administration has been shown to be related to a decreased oxidative load, increased NO concentration and increased cGMP levels in smooth muscle cells (Anwar *et al*., 2011). Chronic administration of melatonin has also been shown to be advantageous in blood pressure control. In patients with metabolic syndrome (MS), two months treatment with melatonin lowered systolic and diastolic blood pressure in human volunteers. The reduction in blood pressure was attributed to the activation of eNOS and increase in NO synthesis (Kozirog *et al*., 2010). Melatonin also reduced blood pressure in adult spontaneously hypertensive rats with established hypertension after a six week treatment period, a result linked to enhanced NOS activity and reduced ROS (Pechánová *et al*., 2007).

The increase in mean systolic pressure in the water control and RUF treatment groups could be explained by a relative lack in handling these groups. Rats not receiving daily subcutaneous injections were subject to less handling procedures when compared to rats receiving daily injectable treatments. It has been shown that rat handling and cage change are capable of causing an elevated heart rate and blood pressure levels for up to five hours (Meller *et al*., 2011). Thus, in future studies, additional care should be given to equalise handling procedures between groups. However, mean diastolic pressure and mean arterial pressure were not increased in the water control and RUF treatment groups, while the RF group did not present with an increase in blood pressure when compared to any of the other groups. As previously mentioned in the results chapter, it is important to note that blood pressure measurements had to be discontinued during the course of the animal studies due to the unanticipated renovations at the Central Animal Housing Unit of the Faculty of Medicine

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and Health Sciences, which resulted in high noise levels and interruptions that were not conducive to performing proper blood pressure measurements on the animals. This resulted in incomplete measurements for some of the treatment groups, and no measurements for the Mel and NRF groups.

4.3.3. Vascular Function

In our experimental setting, aortic rings harvested from the nicotine treatment group showed a significant pro-contractile response to Phe administration when compared to the veh control group, however, no significant effects were observed in response to ACh-induced vasorelaxation compared to the veh control group (Fig 3.13, Fig 3.14). Our data also show that aortic rings harvested from Mel treatment rats demonstrated a significantly greater anti-contractile response when compared to the water control, RF and RUF treatment groups (Fig 3.15). In terms of the ACh-induced relaxation studies, aortic rings from the Mel, RF and RUF groups showed a significant pro-relaxation response when compared to the water control (Fig 3.16). In the co-treatment experiments, melatonin, RF and RUF co-treatment induced significant anti-contractile responses when compared to the nicotine treatment group. Furthermore, aortic rings harvested from the NMel group also showed additional anti-contractile responses when compared to NRF and NRUF groups (Fig 3.17).

Pro-contractile and anti-relaxation effects of nicotine in aortas have previously been demonstrated, where oxidative damage, resulting in impaired integrity of the vascular endothelium was suggested as possible mechanism of action (Chakkarwar, 2011, Tao *et al*., 2013, Şener *et al*., 2005). Blood vessels undergo adaptive remodelling (angioadaptation) in response to various hemodynamic stimuli and can contribute to the pathophysiology of CVD (Pries *et al*., 2005). Chronic nicotine administration has been shown to cause important changes to the cytoarchitecture of rat aortas. Changes include injury of the endothelium, loss of elastic properties, as well as an increase in the amount of connective tissue (Rodella *et al*., 2010). Aortic remodelling in a rat model of chronic nicotine administration has been examined by Zainalabidin *et al* (2014). Nicotine treated rats showed an increase in blood pressure and pulse pressure when compared to the control. Aortas from nicotine treated rats showed an increase in tunica media thickness and decrease in lumen diameter, vascular remodelling properties which could help to explain nicotine-induced hypertension.

In a previous study where chronic nicotine treatment in Wistar rats was shown to exert a pro-contractile response to Phe administration, as well as reduced endothelium-dependent relaxation response to ACh administration in vascular contraction / relaxation experiments, nicotine administration was also associated with an increase in lipid peroxidation and tissue

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collagen content of aortic tissue (Şener *et al*., 2005). The pro-contractile response could be attributed to vascular remodelling associated with chronic nicotine exposure, such as loss in elasticity, increase in connective tissue and decrease in lumen diameter. These factors also underlie the increase in blood pressure, as seen in mean systolic, mean diastolic and mean arterial pressure in the nicotine group when compared to the veh control.

Even though it was not necessarily expected that melatonin and rooibos would improve the vascular function when compared to an untreated control, it could indicate that both melatonin and rooibos have inherent antioxidant potential. These findings could also suggest that melatonin and rooibos are capable of activating eNOS, thereby increasing the bioavailability of NO.

Pro-relaxation findings in the nicotine co-treatment groups (NMel and NRF) indicate that melatonin and fermented rooibos were able to counter-act the harmful effects of nicotine on vascular function. ACh relaxation is dependent on eNOS activation and the subsequent release of NO, which diffuses into underlying smooth muscle cells, leading to vascular relaxation. Thus, the significant pro-relaxation responses of the NMel and NRF groups could be indicative of enhanced eNOS activation, resulting in an increase in the release of vasoactive NO.

The modulating effects of rooibos treatment in a model of nicotine-induced vascular injury have, to our knowledge, not previously been investigated by means of isometric tension studies in vascular ring segments. The data on the vascular effects of rooibos presented in this dissertation are therefore novel. While studies into the modulating effects of rooibos in terms of vascular contraction and relaxation are lacking, our results could be explained by its flavonol content. The flavonol content of rooibos underlies its antioxidant and anti-inflammatory activities, and could also help to explain its vaso-relaxant activities (Chan *et al*., 2000, Perez-Vicaino *et al*., 2006).

On the other hand, the modulating effects of melatonin on the contraction / relaxation of aortas excised from rats receiving chronic nicotine administration have previously been demonstrated. Melatonin administration in nicotine-treated animals restored vascular function and lead to a reduction in tissue damage in the bladder and aorta, which was associated with a significant reduction in lipid peroxidation (Şener *et al*., 2004). Chronic melatonin treatment has previously been shown to improve the ACh-induced relaxation response in normotensive Wistar-Kyoto (WKY) rats, when compared to untreated controls. It was suggested that melatonin could enhance endothelium-dependent vasodilation, possibly due to an enhancement of the vascular NOS pathway (Girouard *et al*., 2001).

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The first pro-relaxation action of melatonin in aortic ring studies was demonstrated in rabbit aorta. Melatonin was capable of inhibiting contractile responses to 5-hydroxytryptamine (5-HT), whereby increased levels of cGMP was proposed as a possible mechanism of action (Satake *et al*., 1991a). A subsequent study in rat aortas confirmed the vaso-relaxing response of melatonin to 5-HT. Removal of the endothelium partially inhibited the vaso-relaxing effect of melatonin, indicating the endothelium dependent action of melatonin (Satake *et al*., 1991b). Chronic melatonin treatment was shown to reduce blood pressure and improve ACh-induced relaxation in aortas from spontaneously hypertensive rats, where an improved vascular NOS pathway activity was suggested as mechanism for hypotensive and pro-relaxation effects of melatonin (Girouard *et al*., 2001). The ability of melatonin to decrease Phe-induced vasoconstriction and its pro-relaxation response to ACh was also demonstrated in aortas of streptozotocin-diabetic rats (Reyes-Toso *et al*., 2002).

Melatonin is a potent free radical scavenger (Tan *et al*., 1993) that has been shown to reduce the degree of histological aorta damage, which is associated with nicotine use, including damage in the tunica intima and tunica media (Rodella *et al*., 2010a). It was proposed that melatonin's beneficial effects on nicotine-induced vasculopathy could be attributed to blocking the activation of extracellular signal regulated kinase (ERK) and other signalling pathways in which the enzyme is involved (Rodella *et al*., 2010b).

As far as we are aware, the effects of fermented rooibos as treatment for nicotine induced injury remain unexamined in *in vitro* models of rat aortic endothelial cells. Our data show that nicotine was capable of reducing NO production in AECs (Fig 3.19), which was associated with increased cell death, both in terms of necrosis and apoptosis (Fig 3.20, Fig 3.21). Nicotine has been widely used in *in vitro* studies and for a wide variety of applications and injury states (Tonnessen *et al*., 2000) where cell types ranged from smooth muscle cells (Jacob-Ferreira *et al*., 2010) to human aortic endothelial cells (HAECs) (Alamanda *et al*., 2012), and human umbilical vein endothelial cells (HUVECs) (Albaugh *et al*., 2001). Nicotine treatment has been shown to induce injury in different cell types. In endothelial cell models, nicotine treatment resulted in changes to endothelial cell surface intercellular adhesion molecule (ICAM) expression in HUVECs (Speer *et al*., 2002). In cultured human endothelial cells, nicotine increased endothelin production (Lee and Wright, 1999) and induced E-selectin transcription in HAECs (Alamanda *et al*., 2012). Chronic nicotine treatment has been shown to decrease NO release, which correlated with a significant downregulation in eNOS gene expression in HUVECs when compared to control (Park *et al*., 2011). Nicotine has also been shown to invoke endothelial cell apoptosis in HUVECs (Wang *et al*., 2011) and inhibit cell proliferation, which is suggestive of cytotoxicity (An *et al*., 2014).

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Based on the significant anti-contractile and pro-relaxation responses observed with RF co-treatment in our aortic ring experiments, nicotine-treated AECs were pre-treated with RF to determine if it could affect the injury model. Pre-treatment of AECs with RF significantly improved NO production in nicotine-injured cells (Fig 3.24), however necrosis was unaffected (Fig 3.25). The ability of RF to counter-act the deleterious effects of nicotine on NO production support the findings observed in aortic rings and indicate the activation of eNOS as a possible underlying mechanism to improved vascular function seen in the RF group. As far as we are aware, these data are novel.

Rooibos and some of its compounds have recently been examined in human umbilical vein endothelial cells (HUVECs) and under varying conditions. Aspalathin and nothofagin, the two major dihydrochalcones found in unfermented rooibos have been investigated for their anti-inflammatory effects. Aspalathin and nothofagin were both found to inhibit lipopolysaccharide (LPS)-induced barrier disruption, expression of cell adhesion molecules and adhesion and migration of neutrophils to human endothelial cells (Lee and Bae, 2015). Aspalathin and nothofagin have also been used to demonstrate their inhibitory effects on endothelial protein receptor shedding in HUVECs (Kwak *et al*., 2015).

4.3.4. Antioxidant Enzyme Activity and Oxidative Stress Status

SOD is regarded as one of the primary defences against ROS and is responsible for the conversion of $O_2^{\bullet-}$ to H_2O_2 and O_2. Accumulation of H_2O_2 leads to the up-regulation of antioxidant enzymes CAT and GPx (Nelson *et al*., 2006, Goyal and Basak, 2010). An increase in SOD activity can thus be regarded as a crucial first step in the antioxidant defence mechanisms, by enhancing oxidative stress signals that stimulate other antioxidant enzymes (Vouldoukis *et al*., 2004, Cloarec *et al*., 2007). CAT accelerates the decomposition of H_2O_2 to H_2O (Li *et al*., 2014), while GPx reduces H_2O_2 to H_2O and lipid hydroperoxidases to alcohols (Lubos *et al*., 2011). It is important to note that ROS can reduce and eliminate the protective abilities of NO, highlighting the importance of an improvement in antioxidant capacity in the presence of nicotine.

The mitochondria supply approximately 90% of the ATP required by the myocardium, which is a highly energy demanding tissue. Under physiological conditions up to 2% of oxygen can be converted to $O_2^{\bullet-}$ mainly at complex I and III of the electron transport chain (ETC) (Boveris and Chance, 1973). To combat ROS production, mitochondria rely on detoxifying enzymes and non-enzymatic antioxidants, of which MnSOD is a first line defence. MnSOD converts $O_2^{\bullet-}$ into H_2O_2, which can be detoxified further by GPx and peroxiredoxine (Prx-III) (Andreyev *et al*., 2005). It is also possible for $O_2^{\bullet-}$ to be released in the membrane space, where

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(CuZnSOD) causes conversion to H_2O_2, as well as for cytochrome *c* to scavenge $O_2^{\bullet-}$ which is leaked into the intermembrane space (Pasdois *et al*., 2011). Since mitochondria are a major site for the generation of ROS, dysfunction in mitochondria can increase the risk for a number of diseases, including CVD (Ajith and Jayakumar, 2014). The removal of dysfunctional mitochondria through autophagy is important in the maintenance of cell viability, however, prolonged over-production of ROS can cause a decline in the removal process, which could be involved in the development of CVD (Dutta *et al*., 2012).

SOD is found in high concentrations in the heart and liver. Different types of SODs have been characterized according to their metal content, of which copper-zinc-containing SOD (CuZnSOD) is found in the cytosol and manganese-containing SOD (MnSOD) in the mitochondria (Marklund, 1980). Since mitochondria are abundant in cardiac tissue, it was decided to examine the SOD activity of heart cytosolic and heart mitochondrial fractions separately.

In the heart cytosolic fraction, the SOD activity of the RF treatment group was significantly increased when compared to the water control, RUF, Mel, NRF and NMel treatment groups. However, SOD activity in the heart mitochondrial fraction of the RF group was significantly reduced when compared to the water control, Mel, NRF, and NRUF groups. The SOD activity of the NRUF group was also significantly increased in the heart mitochondrial fraction, when compared to the veh control group (Fig 3.26, Fig 3.27). In liver tissue homogenates, the SOD activity of the RF treatment group was significantly increased compared to the water control, Mel and nicotine groups. The SOD activity of the NRF and NMel groups were also significantly increased compared to the nicotine group, indicating an improvement in antioxidant capacity (Fig 3.28).

No significant differences were found between the nicotine treatment group and the veh control group in either fraction of heart tissue homogenates, however SOD activity was significantly reduced in the nicotine treatment group of liver tissue homogenates when compared to the veh control group (Fig 3.26, Fig 3.27, Fig 3.28).

CAT activity was significantly reduced in the nicotine treatment groups in both heart cytosolic fraction and liver tissue, when compared to veh controls. In the heart cytosolic fraction, CAT activity was increased in the NRUF and NMel groups, as well as RF and Mel groups when compared to the nicotine treatment group (Fig 3.29). CAT activity was significantly increased liver tissue homogenates in the Mel, NRF and NMel treatment groups, when compared to the nicotine treatment group (Fig 3.30). However, no significant differenced were found between GPx activity of the respective treatment groups (Fig 3.31).

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Nicotine treatment has previously been shown to decrease SOD activity in both liver and heart tissue, when compared to untreated controls (Gumustekin *et al*., 2010, Neogy *et al*., 2008). Similarly, CAT activity has previously been shown to be reduced by nicotine treatment in male Wistar rats, when compared to untreated controls (Neogy *et al*., 2008). The increased SOD activity in the RF treatment group in both heart cytosolic tissue and liver tissue, when compared to the water control group could be attributed to the pro-antioxidant properties of rooibos. However, the SOD activity in heart mitochondrial tissue of the RF group was significantly reduced compared to several other groups, including the water control, which is in contrast to findings in heart cytosolic tissue and liver tissue. Signalling pathways associated with ROS are complex and compartmentalized, which could explain why findings are not necessarily consistent between different tissue fractions (Brown and Griendling, 2015). In addition, the bioavailability of rooibos and its various polyphenolic constituents and their exact mechanisms of action have not been fully elucidated (Stalmach *et al*., 2009, Courts and Williamson, 2009).

In our experimental setting, RF increased SOD activity in the heart cytosolic fraction and liver tissue, indicating enhanced antioxidant capacity, which is a hallmark of rooibos. In liver tissue, co-treatment of nicotine with RF and Mel (NRF and NMel groups) showed significantly increased SOD activity when compared to the nicotine treatment group. Furthermore, CAT activity in liver tissue of the NRF and NMel groups were also increased, which correlates with the increase observed in SOD activity.

Rooibos has been shown to have the potential to increase the activity of antioxidant enzymes, including SOD and GPx, in diabetic rats (Ayeleso *et al*., 2014). A four week rooibos treatment period was able to attenuate SOD, CAT and GPx activities in rat brain extracts in an immobilization stress model (Hong *et al.*, 2014). Rat serum SOD levels were increased after a four week treatment with rooibos, when compared to untreated controls, an action attributed to the flavonoid content in rooibos. Furthermore, SOD levels were significantly higher in rooibos treated animals, using a rat colitis model. In the same study, 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in urine were significantly decreased in the rooibos treatment group when compared to controls, suggesting that rooibos is effective in reducing DNA damage caused by oxidative reactions (Baba *et al*., 2009). The composition of fermented rooibos and unfermented rooibos differ in terms of flavonol content, which could lead to different modulating capacities. However, investigations comparing the modulating effects of fermented rooibos to unfermented rooibos are limited.

Melatonin has previously been shown to increase SOD activity in both the liver and lung of nicotine-treated rats over a treatment period of four weeks (El-Sokkary *et al*., 2007). Melatonin

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also increased SOD activity in a fructose-induced model of metabolic syndrome in a rat model. However, CAT activity was unchanged in all treatment groups after the eight week treatment period (Demirtas *et al*., 2015). In a rat model of renovascular hypertension, a nine week treatment period with melatonin improved cardiovascular function, which was associated with an increase in SOD and CAT activity (Ersahin *et al*., 2009). Melatonin was also shown to have a protective role in myocardial oxidative damage in a mercury-induced murine model, which was associated with an increase in SOD activity (Jindal *et al*., 2010).

TBARS and CD levels are both indicators of lipid peroxidation. Lipid peroxidation leads to the rearrangement of polyunsaturated fatty acid double bonds, leading to the formation of CD, which indicates primary lipid peroxidation, while measurement of TBARS levels is the method most frequently used to determine lipid peroxidation and indicates secondary lipid peroxidation. In the current study, analysis of CD levels did not result in any significant difference between treatment groups. TBARS levels were increased in the nicotine treatment group and co-treatment with melatonin (NMel group) significantly reversed TBARS levels, indicating a reduction in oxidative stress (Fig 3.33). Nicotine has previously been shown to increase lipid peroxidation (MDA levels) in Sprague-Dawley rats, when compared to untreated controls in a study which investigated the effects of vitamin E and an extract of *Hippophea rhamnoides* on nicotine-induced oxidative stress (Gumustekin *et al*., 2010), thus supporting our findings.

Overall, the above data support the findings observed in the aortic ring studies (section 2.3), whereby RF and Mel were capable of modulating the harmful effects of nicotine in vascular function (NRF and NMel groups vs nicotine group). An increase in SOD activity and CAT activity (NRF and NMel groups vs nicotine group), reduction in TBARS levels (NMel group vs nicotine group) and anti-contractile and pro-relaxation responses (NRF and NMel groups vs nicotine group) could indicate that RF and Mel were capable of reducing ROS and, in turn, enhancing the release of NO, restoring the balance of the release of vasoactive factors.

4.3.5. Inflammatory Marker (CRP) Levels

CRP is a sensitive, but non-specific marker of the acute inflammatory response and is directly involved in the development of atherosclerosis (Mao *et al*., 2012). Nicotine treatment alone had no effects on serum CRP levels (Fig 3.34). Furthermore, CRP levels remained unchanged in nicotine-treated rats that received RF, RUF and Mel co-treatment. Treatment with RUF and Mel significantly decreased CRP levels compared to water controls. CRP is involved in the proliferation and migration of VSMCs and mediates the uptake of LDL by macrophages, in turn promoting the formation of foam cells (Zwaka *et al*., 2001). Even though it was surprising

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that the CRP levels in the nicotine treatment group did not increase, it has previously been demonstrated that nicotine administration in rats were not associated with an increase in CRP levels (Cecconi *et al*., 2008). It is difficult to explain why nicotine treatment had no effect on CRP levels in the present study. Unfortunately, the serum IL-6 and TNF-α levels were not available in view of the fact that the concentrations in the samples supplied to the CPGR were below the detectable range as reported to us. This is unfortunate, as information on the levels of IL-6 in particular would have been helpful to support the CRP results, since IL-6 is responsible for CRP induction in the liver (Kushner *et al*., 1995). TNF-α has previously been shown to increase in a model of nicotine-treated human THP1 monocytes, which was suggested to play a role in the progression of atherosclerosis (Zhou *et al*., 2013), and is implicated in the induction of ED (Cardillo *et al*., 2006), possibly due to impairment of endothelium-dependent and NO-mediated vasodilation.

4.3.6. Serum Lipid Measurements

No significant differences were found in TC and PL levels between treatment groups (Fig 3.35, Fig 3.36), however, TG levels were significantly decreased in the nicotine group compared to the veh control, water control, RF and RUF treatment groups (Fig 3.37). TG concentrations were also significantly decreased in the NMel group, when compared to the RUF group. TG levels are typically increased during nicotine exposure (Sudheer *et al*., 2008), while chronic nicotine treatment has been shown to alter lipid metabolism and was associated with an increase in TG levels (Ashakumary and Vijayammal, 1997). It is, however, important to note that the nicotine treatment periods in the aforementioned studies were 22 weeks (154 days) and 90 days respectively, whereas the nicotine treatment period for our current study was six weeks (42 days). These findings could indicate that the effects of nicotine on lipid metabolism develop over time and were not yet apparent after our treatment period.

4.3.7. General Discussion

The increased oxidative stress associated with nicotine use can lead to a reduction in NO bioavailability, impairing endothelium-dependent relaxation. Our results demonstrate that chronically administered nicotine could increase mean systolic, mean diastolic and mean arterial blood pressure, as well as exert pro-contractile effects in isolated aortic rings. The ability of nicotine to impair vascular function and damage the endothelium was further demonstrated and underscored in a model of cultured AECs, where nicotine treatment resulted in a reduction in NO production and increased cell death. In addition to vascular

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damage, nicotine administration leads to reduced SOD activity in liver tissue and CAT activity in both heart cytosol and liver tissue, as well as increased lipid peroxidation, as determined by TBARS levels in serum, all of which suggest that nicotine was capable of inducing oxidative stress, which could underlie the mechanisms leading to a disruption in vascular homeostasis and, in turn, vascular damage. The main findings of the *in vivo*, *ex vivo* and *in vitro* nicotine treatments are summarised in Figure 4.1.

Our results indicate that melatonin and rooibos, specifically RF have the potential to combat nicotine-induced vascular injury. The vascular modulating potential of melatonin has previously been demonstrated (Anwar *et al*., 2011; Kozirog *et al*., 2010; Pechánová *et al*., 2007) and could be attributed to its potent free radical scavenging and antioxidant capabilities (Hardeland *et al*., 1993, El-Sokkary *et al*., 2002), ability to cross biological barriers (Venegas *et al*., 2012) and presence in all bodily fluids, tissues and cellular compartments (Reiter *et al*., 2013), making it an ideal candidate to combat increased oxidative stress. Both melatonin and RF demonstrated the ability to restore vascular function in the *ex vivo* model (anti-contractile and pro-relaxation effects in aortic rings) and increase antioxidant defences in the *in vivo* model (increased SOD and CAT activity). Therefore, based on these findings, it is possible to speculate that melatonin and RF exerted their effects via similar mechanisms. In addition, melatonin was capable of reducing lipid peroxidation (TBARS) in nicotine-treated rats. The effects of RF were further demonstrated in the *in vitro* model of cultured AECs, where RF improved endothelial function (increased NO production) in nicotine-injured cells. The main findings of the *in vivo* and *ex vivo* nicotine and melatonin co-treatments are summarised in Figure 4.2 and the main findings of the *in vivo*, *ex vivo* and *in vitro* nicotine and fermented rooibos co-treatments, as well as the main findings for the *in vivo* and *ex vivo* nicotine and unfermented rooibos co-treatments are summarised in Figure 4.3.

It is interesting to note that rooibos (RF and RUF) exerted modulating effects in nicotine-induced vascular injury to varying degrees. Epidemiological evidence suggests that dietary-derived antioxidants have potential for disease prevention (Froman *et al*., 2014) and it has been shown that dietary polyphenols can increase endothelium-dependent NO generation by modulating cellular sensors for oxidative stress. NO is capable of reacting with $O_2^{\bullet-}$ to form peroxynitrite, which can lead to a nuclear accumulation of nuclear factor erythroid 2-related factor (Nrf2) (Mann *et al*., 2007). Nrf2 is a redox sensitive transcription factor, involved in antioxidant response element (*ARE*) dependent gene expression (Nguyen *et al*., 2003). Under conditions of oxidative stress, Nrf2 is capable of activating *ARE* dependent transcription of phase II and antioxidant defence enzymes, such as glutathione-*S*-transferase, GPx and heme-oxygenase-1 (Kensler *et al*., 2007). In addition, the safety of both fermented and unfermented rooibos has previously extensively been investigated in a rat model and no

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adverse effects were found. Parameters examined included body weight, liver weight and liver and kidney parameters, including total cholesterol, aspartate transaminase and creatinine (Marnewick *et al*., 2003).

The particularly promising modulating effects of RF when compared to RUF could be attributed to the presence of ferulic acid in RF, since no ferulic acid was detected in RUF. Ferulic acid is a potent antioxidant and free radical scavenger (Mancuso and Santangelo, 2014) and has been shown to possess blood pressure lowering effects (Suzuki *et al*., 2002). It has been proposed that ferulic acid has multifactorial vasodilating effects, involving the reduction of angiotensin II and activation of eNOS, leading to an increase in NO (Suzuki *et al*., 2007). RF also had a significantly higher flavonol content, when compared to RUF, which translated to a higher daily intake of flavonols in the RF and NRF treatment groups. The higher flavonol content in RF could also help to explain the greater modulating capacity of fermented rooibos. These findings suggest that both RF and RUF have the potential to restore vascular homeostasis, and in turn, modulate the harmful effect of nicotine in the context of vascular injury. RF specifically presents potential modulating effects, as demonstrated by its anti-contractile and pro-relaxation effect in aortic rings, and supported by *in vitro* findings.

It is possible that melatonin and rooibos exert their effects through different mechanisms, all of which would result in a restoration of vascular homeostasis and, in particular, the function of NO.

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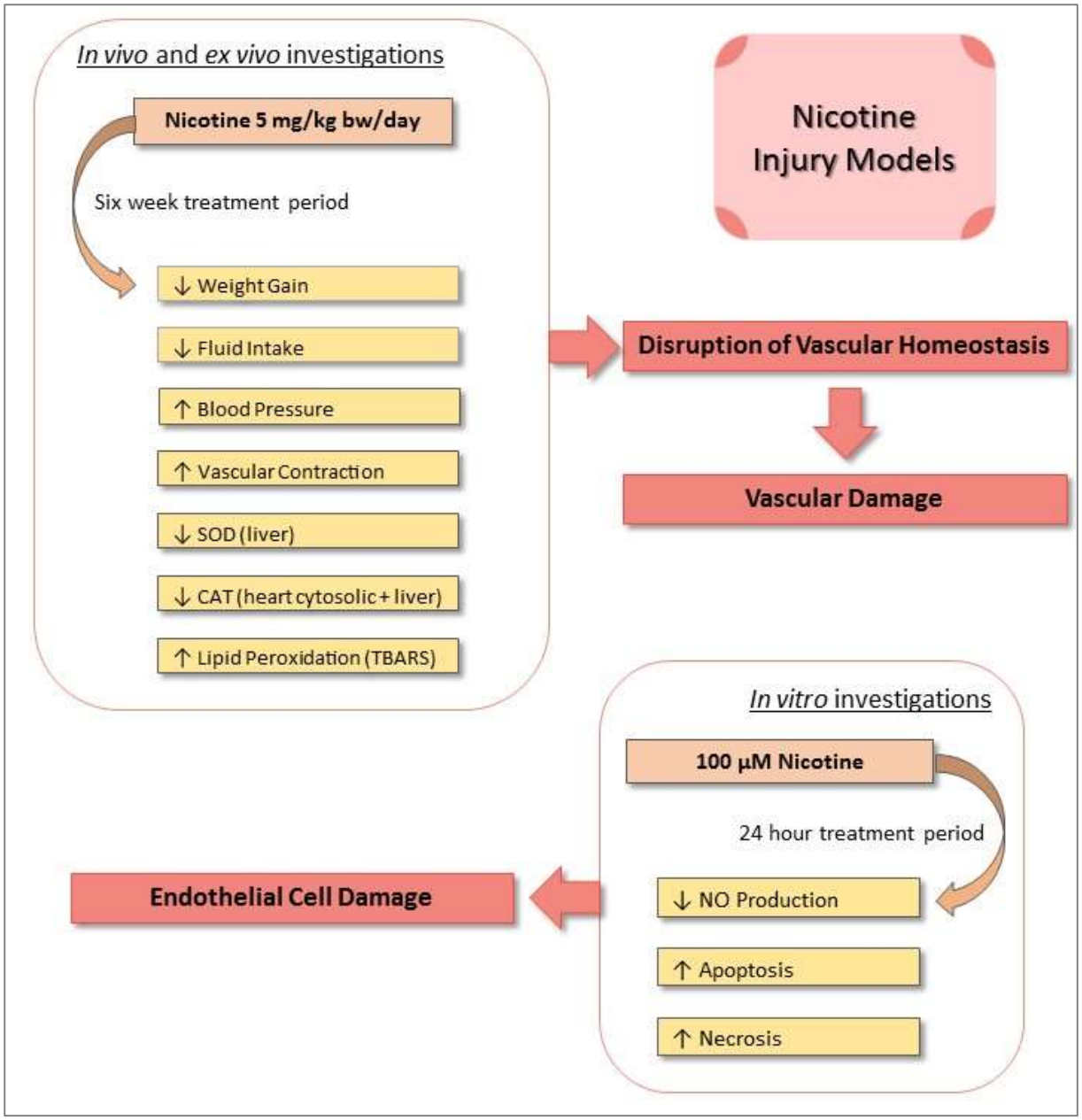

Figure 4.1. Summary of findings of *in vivo*, *ex vivo* and *in vitro* nicotine treatment.

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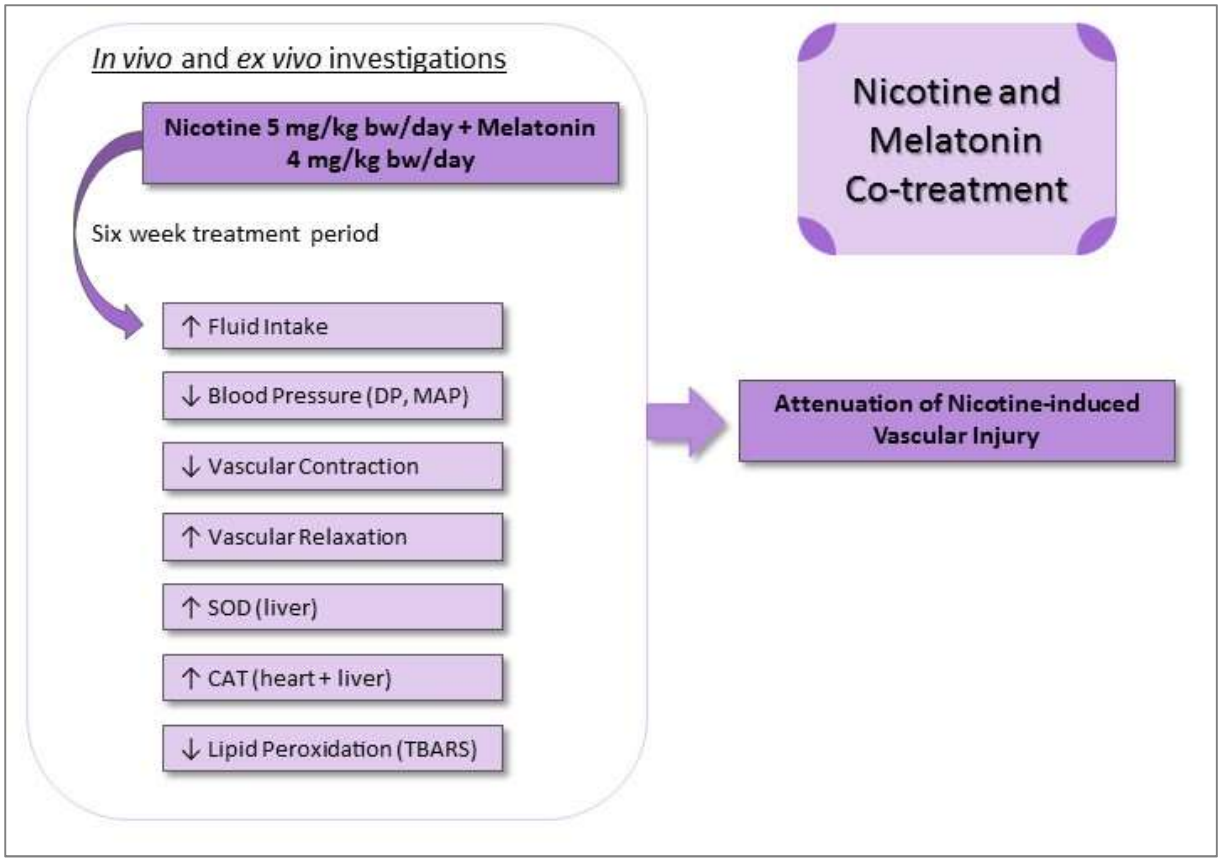

Figure 4.2. Summary of *in vivo* and *ex vivo* findings of nicotine and melatonin co-treatment.

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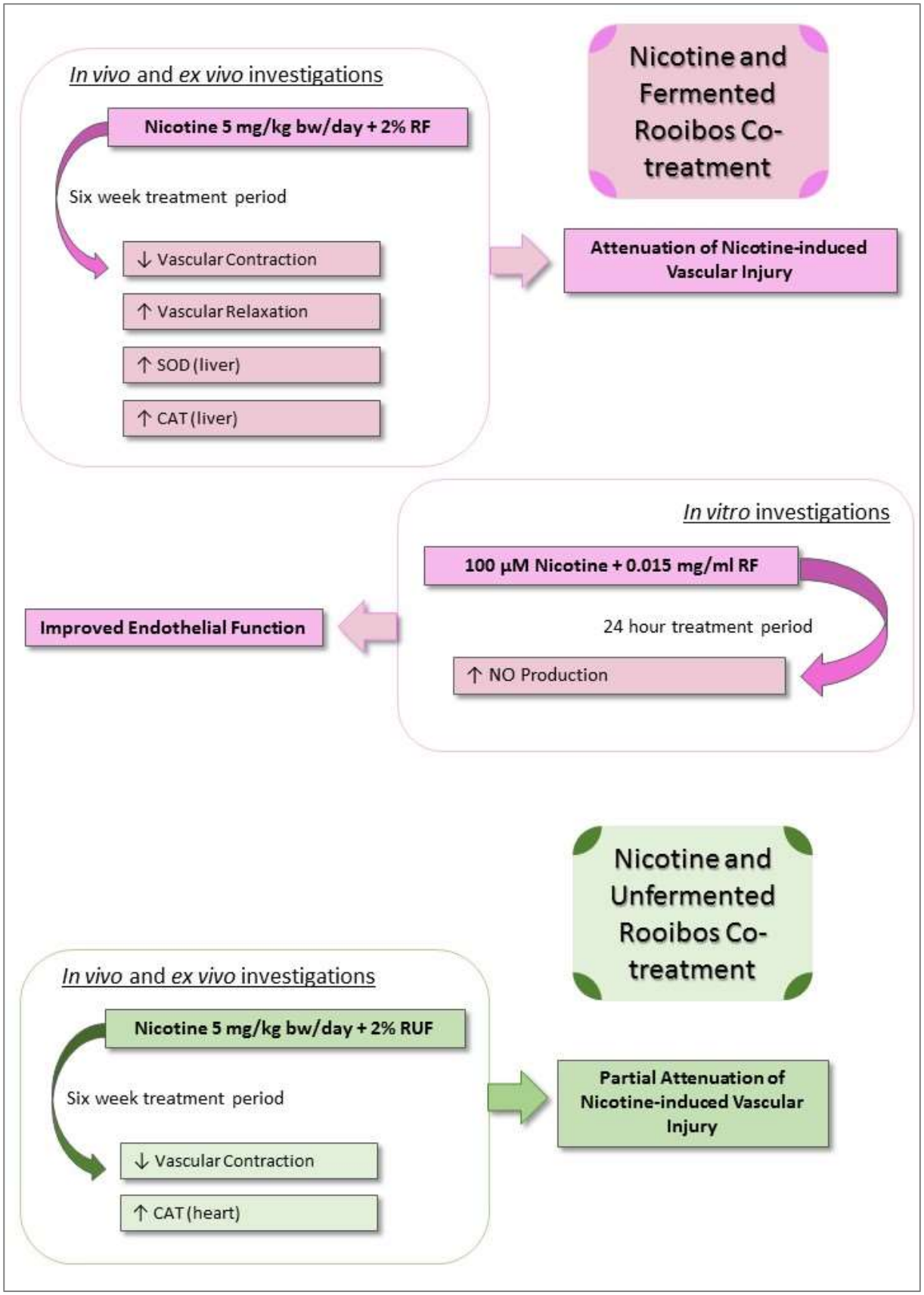

Figure 4.3. Summary of findings of *in vivo*, *ex vivo* and *in vitro* nicotine and fermented rooibos co-treatment and *in vivo* and *ex vivo* nicotine and unfermented rooibos co-treatment.

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CHAPTER 5: CONCLUSION

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5.1. General Conclusion

This study aimed to investigate the modulating effect of rooibos (fermented and unfermented) and melatonin in the context of nicotine-induced vascular injury. Oxidative stress can lead to a loss of vascular homeostasis, in particular a reduction in the bioavailability of NO, which can lead to the development of ED. Nicotine exposure is associated with early endothelial changes, such as endothelial dysfunction, which is an important precursor of atherosclerosis and, in turn, cardiovascular disease. Therefore, it is important to identify therapeutic compounds capable of modulating oxidative stress injury in the context of vascular health, in order to reduce or reverse endothelial dysfunction and, in turn, restore vascular homeostasis. Both rooibos and melatonin have been shown to possess antioxidant effects. Rooibos has also been shown to have immune-modulating and chemo-protective actions and is capable of improving the lipid profile and redox status, while melatonin is involved in many physiological processes, including blood pressure control, free radical scavenging and has been demonstrated to be cardioprotective. In order to examine the modulating effect of rooibos and melatonin, this study utilised an *in vivo* model of male Wistar rats and an *ex vivo* model of rat aortic ring segments, complemented by an *in vitro* model of cultured aortic endothelial cells.

5.1.1. Nicotine-induced Injury

Firstly, an *in vivo* model of nicotine-induced vascular injury was established in male Wistar rats. Concentrations of 1 mg/kg bw/day nicotine and 2 mg/kg bw/day nicotine did not result in sufficient vascular injury in our experimental setting, with a reduction in fluid intake being the only endpoint significantly influenced in nicotine treated groups when compared to the veh control group. Based on these findings, a higher nicotine concentration, namely 5 mg/kg bw/day nicotine, was utilised over a six week treatment period. Nicotine treatment significantly influenced biometric parameters and fluid consumption in the animals. Nicotine increased mean systolic, mean diastolic and mean arterial pressure and exerted a pro-contractile response in aortic rings, as determined *ex vivo* isometric tension studies. The apparent harmful vascular effects of nicotine were further underscored in an *in vitro* model utilising adult rat aortic endothelial cells, whereby a 24 hour incubation period with 100 µM nicotine leads to reduced intracellular NO levels and increased cell death (necrosis and apoptosis). Furthermore, nicotine administration resulted in a decrease in SOD activity in liver tissue and decrease in CAT activity in both heart and liver tissue, as well as an increase in lipid peroxidation as indicated by TBARS determination in serum. Based on these results, 5 mg/kg bw/day nicotine, subcutaneously administered over a six week treatment period, resulted in

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significant vascular and endothelial injury, which was associated with increased oxidative stress and reduced antioxidant activity.

5.1.2. Modulating Capabilities of Rooibos (Fermented and Unfermented) and Melatonin

Both melatonin and rooibos were capable of restoring nicotine-induced changes in certain endpoints of vascular function and increasing antioxidant activity. However, fermented and unfermented rooibos showed varying degrees of modulation, with the restorative effect of fermented rooibos being more pronounced and comparable to the capabilities of the potent free radical scavenger and antioxidant, melatonin. This was demonstrated by an *ex vivo* model of vascular function, as well as *in vivo* model indicating an increase in antioxidant defences. Additionally, melatonin also decreased lipid peroxidation (TBARS), when compared to the nicotine treatment group. These findings were further substantiated by *in vitro* investigations, where pre-treatment of AECs with RF was capable of restoring NO production in nicotine-injured cells. As far as we are aware, this is the first study to examine the effects of rooibos in a model of nicotine-induced injury in AECs. Unfermented rooibos treatment resulted in the modulation of nicotine-induced vascular injury to a lesser degree compared to fermented rooibos, and was capable of exerting anti-contractile responses in aortic rings when compared to the nicotine treatment group. Furthermore, treatment with unfermented rooibos also increased CAT activity in heart tissue of nicotine-treated animals.

Melatonin's modulating capabilities could be attributed to its antioxidant and free radical scavenging capabilities. Melatonin is able to easily cross biological barriers and is present in all bodily fluids, tissues and cellular compartments, which could explain its modulating effects in different tissues and across different endpoints. In addition to its potent antioxidant properties, other possible mechanisms of action of melatonin include the inhibition of ERK activation, reduction in histological aorta damage and enhanced endothelium-dependent vasodilation, which would indicate improved vascular NOS pathway activity. All of the abovementioned mechanisms would result in restoring vascular homeostasis and, in particular, the release of NO.

The finding that fermented rooibos was capable of greater modulation of vascular injury was somewhat unexpected, since two major dihydrochalcones, namely aspalathin and nothofagin, are greatly reduced during the fermentation process. However, the analysis of the aqueous rooibos preparations indicated that fermented rooibos had a higher flavonol content, which translated to a higher daily flavonol intake in both the RF and NRF treatment groups. This finding could, in part, help to explain the greater modulating capacity of fermented rooibos. It is possible that rooibos exerts its modulating effects through the transcription factor Nrf2,

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which has been shown to activate *ARE* dependent transcription of phase II antioxidant defence enzymes, which would combat oxidative stress in order to restore vascular homeostasis. The presence of ferulic acid in fermented rooibos (as shown in the HPLC analysis) could explain why fermented rooibos was capable of exerting a greater modulating effect than unfermented rooibos, which contained no detectable ferulic acid levels. Ferulic acid has a multifactorial vasodilating effect, whereby angiotensin II is reduced and eNOS activated, leading to an increase in NO production. It should also be considered that antioxidants administered in high concentrations may not be beneficial and can exert pro-oxidant effects, which could help to explain the lesser modulating capability of unfermented rooibos when compared to fermented rooibos in the model of nicotine-induced vascular injury. However, studies into the bioavailability of rooibos, specifically fermented rooibos versus unfermented rooibos, are lacking.

5.2. Novel Findings Arising from this Study

The investigations pertaining to the effects of rooibos on nicotine-induced vascular injury have not previously been demonstrated. The investigations of the current study revealed the following novel findings, particularly with regard to fermented rooibos:

- Improved vascular function as determined by vascular contraction / relaxation experiments.
- Increased NO production in nicotine-injured rat AECs.
- Improved antioxidant enzyme activity as determined by SOD and CAT.

5.3. Final Conclusions

Both fermented rooibos and melatonin have the potential to modulate nicotine-induced vascular injury and present attractive candidate therapeutic strategies for reducing or reversing early endothelial injury, and, in turn, possibly future CVD. While the modulating capabilities of rooibos and melatonin can be linked to their antioxidant properties, it is possible that they function through different mechanisms in order to exert their effect. However, eNOS activation and subsequent release of NO could underlie the modulating actions of both rooibos and melatonin. Rooibos and melatonin have both been found to have no adverse effects; in addition, fermented rooibos is readily available to consumers and could provide a cost-effective treatment to reduce cardiovascular risk when using nicotine.

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5.4. Shortcomings of the Study

- Blood pressure measurements could not be completed for all treatment groups, due to the unexpected renovations undertaken at the animal housing facility.
- No western blotting analysis of aortic rings performed, which could have supplied more information on the underlying cellular mechanisms of the different treatment groups. Proteins of interest that would have added value to our understanding of underlying mechanisms, include eNOS (main enzyme responsible for endothelial production of nitric oxide), PKB/Atk (cell growth and survival protein, upstream activator of eNOS, and an important anti-apoptosis protein) and p22phox (marker of NADPH-oxidase activity – an important vascular source of ROS and oxidative stress).
- *In vitro* investigations into the effect of melatonin on nicotine-injured rat AECs would have supplied valuable insights into their cellular mechanisms; these studies could, however, not be undertaken due to time and financial constraints.

5.5. Future Directions

Further investigations into this topic are partially based on the shortcomings of this study and would include the following:

- Western blotting analysis of aortic rings as explained above.
- Proteomic analysis of aortic rings to explore large-scale protein expression patterns and differential protein regulation that could greatly contribute to a better understanding and identification of novel cellular pathways and mechanisms involved in vascular injury and protection.

In addition, the following investigations should be considered:

- Examination of histomorphological changes in aortas, including tunica media thickness and lumen diameter, would help to explain changes in blood pressure and vascular contraction / relaxation investigations.
- The combination of rooibos and melatonin as treatment for nicotine-induced vascular injury provide a further investigation opportunity. Since it is possible that rooibos and melatonin exert their antioxidant, and indeed vascular modulating effects, through different mechanisms, it is possible that co-treatment of rooibos and melatonin in the case of nicotine-induced vascular injury could further augment their modulating capabilities.

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- Investigations to determine whether nicotine or nicotine in combination with rooibos or melatonin could influence intracellular calcium levels in endothelial cells.

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5.6. Outputs

The following outputs were generated from the study:

5.6.1. Conference Contributions

Presentations:

Smit-van Schalkwyk M, Windvogel SL, Strijdom H. **Rooibos and melatonin as prospective therapies for nicotine induced effects on vascular function: injury models and preliminary findings.** 42nd Annual Conference of the Physiology Society of Southern Africa; 2014.

Posters:

Smit-van Schalkwyk M, Windvogel SL, Strijdom H. **The effects of rooibos (*Aspalathus linearis*) and melatonin on vascular function in a rat model of nicotine-induced vascular injury.** 59th Annual Academic Day, Faculty of Medicine and Health Sciences, Stellenbosch University; 2015.

5.6.2. Postgraduate Student Supervision

BSc Honours students:

- Mnr Kevin Els – HonsBSc (Medical Physiology) – Graduated December 2014
- Ms Zimvo Maqeda – HonsBSc (Medical Physiology) – to graduate December 2015

5.6.3. Successful Applications for Research Grants

This project was successful in obtaining research grants from the following funding bodies:

- Harry Crossley Foundation, Stellenbosch University: 2014 – 2015

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CHAPTER 6: REFERENCES

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“Three things that never come back:

the spent arrow;

the spoken word;

the lost opportunity.”

William George Plunkett